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THIRTIETH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

RICHMOND, VIRGINIA, DECEMBER 27, 28, 29, 1928

Headquarters, Hotel Jefferson

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GENERAL BACTERIOLOGY

1. Dissociation of Acido-proteolytic Bacteria by the Climbing Culture Method. PROF. DR. CONSTANTINO GORINI, R. Scuola Superiore D'Agricoltura, Milan, Italy.

The acido-proteolytic bacteria, discovered by me in 1892–1894[1] attack both carbohydrates and proteins simultaneously. They are able, therefore, to peptonize even in an acid medium.

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[1] Atti dei Laboratori Sanità Publica al Minist. Interni, Roma, 1892. Revista d'Igiene e Sanità Publica, Roma, 1893, **4**, 549; Hyg. Rundschau, 1893, **3**, 381; Giornale della reale Soc. ital. d'Igiene, 1894, **16**, No. 4.

The climbing culture method, proposed and described by me in 1903[2] consists in the inoculation of the condensation water of a sloped tube of agar with bacteria which have a tendency to grow as a spreading film on the surface of the medium (*B. proteus*, *B. typhi*, *B. tetani*, etc.). By this method these bacteria may be easily isolated from diverse materials.

Among the acido-proteolytic bacteria described by me in 1904[3] is *Bacillus acidificans-presamigenes-casei*. This is a spore-bearing bacillus similar in form to *B. subtilis*. This organism exhibits physiological mutations, having some cells which are predominantly proteolytic and others which are predominantly acidifiers.[4] Two types of colonies corresponding to these two types of processes are found on gelatin plates. A granular type has been called the unstable S type, and a filamentous type has been called the stable R type.[5]

Having observed that the granular type is much more motile than the filamentous type, I have applied the climbing culture method to cultures of *B. acidificans-presamigenes-casei*. In this way I have obtained the isolation of the granular type, making a series of transfers from the top of the spreading edge of the growth.

From this I conclude that my climbing-culture method may be employed not only for the isolation of motile or spreading species, but also for the microbic dissociation of species whose cells have a motile phase in their evolution.

2. *The Rate of Growth of a Colony of Bacteria.* L. A. ROGERS AND GEO. R. GREENBANK, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

Growth in a tube filled with nutrient broth does not progress uniformly but by alternate periods of rapid and slow growth.

3. *Physiological Difference Between Young and Old Bacterial Cells.* C. N. STARK AND PAULINE STARK, Cornell University, Ithaca, N. Y.

Differences between young and old bacterial cells have been established through studies of the relative rates of fermentation and the relative resistance to heat of cells of different ages.

[2] Rend. R. Ist. lombardo di Sci. e. Lett., 1903, **36**, 601.

[3] Rend. R. Ist. lomb. di Sci. e. Lett., 1904, **37**, 939; Centralbt. f. Bakt., II Abt., 1906, **16**, 236.

[4] Rend. R. Accad. dei Lincei, 1921, **30**, 312; J. Dairy Sci., 1922, **5**, 510.

[5] Rend. R. Accad. dei Lincei, 1928, **7**, 689; C. r. Acad. Sci., Paris, 1928, **186**, 1070.

The rate of fermentation per cell per hour was calculated by use of the formula

$$X = \frac{S \log \frac{b}{a}}{t(b - a)\ 0.434},$$

where X = amount of acid formed by one cell in one hour

a = number of bacteria at beginning

b = number of bacteria at end

t = time in hours

S = acid formed in time t

It was found that the average rate of fermentation of young cells of *Bacterium coli* was 45.5×10^{-10} milligram acid per cell per hour, while the average rate for old cells in a similar medium and under the same conditions was 9.9×10^{-10} milligram acid per cell per hour.

With respect to heat resistance, it has been shown that the young cells of *Streptococcus fecalis* and 2 types of capsule forming organisms are much more sensitive to heat than are old cells of the same organisms.

4. Bacterial Calorimetry. S. BAYNE-JONES AND HENRIETTA S. RHEES, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

By means of simultaneous observations of the heat production by bacteria and enumeration of the organisms in the culture it is possible to correlate growth curves with curves of the liberation of heat. A combination of such observations, with those of the morphological changes and life-phases of bacteria, yields some information as to the metabolic activities of a culture at various periods. By the use of Buchanan's formula, the rate of heat production by a single bacterial cell can be calculated, and the observed values agree closely with those derived by this calculation.

Changes in the rate of heat production are related to changes in the growth rate of bacteria.

Young bacterial cells produce more heat per cell than older ones.

After about the fifth hour of growth, the production of heat reaches a low level and remains constant, suggesting the existence of a basal metabolic rate during that period.

5. Remarks on Two Modes of Cell Division in Bacteria. GEORGES KNAYSI, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

In the dividing cell of *Mycobacterium tuberculosis*, a sharp clear zone of division, perpendicular to the long axis of the cell, is observed first. The stainable constituents of the protoplasm are drawn back into two dense masses which usually persist at each pole of the daughter cells. The portions of the membrane of the mother cell which limit the clear zone remain for a while until new membranes limit the zone when it withers away. No constriction of the cell at the zone of division was observed during the process.

In the dividing cell of *Proteus vulgaris*, one of the surface granules of the cell membrane grows in and increases in size until it reaches the other side or, more often, two such granules at opposite sides may grow and extend until they meet. At the same time, or sometimes immediately afterwards, the cell becomes more and more constricted at the zone of division. It is probable that the new partition is split by furrowing to form the closing membranes. In the majority of cases the cells remain connected by means of plasmodesma, the chemical nature of which has not yet been ascertained.

It seems, therefore, probable, from the above descriptions and from a close study of the structure of the bacterial cell, that surface tension plays a secondary, if any, rôle in the process of cell division.

6. The Value of Vegetable Extracts in Culture Media. LUTHER THOMPSON, Mayo Clinic, Rochester, Minn.

Extracts were made of potato, carrot, radish, spinach and of beef heart, the latter for comparison. One hundred and fifty grams of grated or ground material were added to 200 cc. of distilled water. The mixture was shaken for ten minutes and then strained and sterilized by filtration.

Potato extract was found to be the most satisfactory of those tried. It gives a total nitrogen of about 1.2 mgm. per cubic centimeter and approximately 0.2 per cent reducing sugars. Amounts as little as 0.01 cc. when added to 6 to 7 cc. of nutrient broth will give growth with many strains of streptococci which will not develop in the broth alone while 0.2 cc. is enough to secure a vigorous growth with nearly all strains encountered.

The growth accelerating factor is not destroyed by heat except when heating causes a precipitate, but is destroyed by the action of certain bacteria. It is thought to be a protein which is easily available to

bacteria, and not a vitamine or growth accessory factor, since similar results may be obtained with the products of bacterial decomposition of peptone.

Potato extract is useful as a substitute for blood in growing streptococci. When added to meat infusion media, it allows the growth of *Hemophilus influenzae*. It may also be used in small amounts as an enriching substance in sugar fermentation tubes instead of serum or meat infusion.

7. *The Radiation of Ultraviolet Light—Mitogenetic Rays, So-Called—By Bacteria and Higher Plants.* RALPH R. MELLON, N. AND E. VON RASHEVSKY, Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh, Pa.

Within recent years Gurvitch has brought forward rather interesting experiments indicating that growing plant tissues emit a short wave length ultraviolet light capable of stimulating cell division and growth enormously. He therefore spoke of the rays as mitogenetic from the large number of mitoses observed in the plants subjected to their action. He worked chiefly with the rootlets of growing onions but also with other plants and embryonic animal tissues. Positive results were so frequent as to lead him to conclude that the phenomenon might be a general one.

The effect would seem to be one of radiation and not electron emanation. This belief is supported by the fact that quartz does not appear to bar its passage, while glass does. Moreover, the radiant energy is capable of diffraction and is absorbed by thick layers of air and by gelatin. Inasmuch as his attempts to photograph the light (?) were failures, he inferred that its wave length lay between 1900 and 2000 A.U. He believed that the gelatin of the photographic plate absorbed the light.

Certain anomalous experiences of our own in connection with the division and differentiation of bacterial cells gave impetus for a photographic demonstration of this phenomenon. If successful in this, it was our purpose to extend the observations to the bacteria. In both of these considerations we have been successful.

We have circumvented the photographic difficulty encountered by Gurvitch by sensitizing the ordinary photographic plate with machine oil, which is known to fluoresce in the presence of ultraviolet light, thus giving a visible light to which the plate is, of course, sensitive. It was found that *B. coli*, as well as growing onions, when exposed to the

sensitized plates for three to five days affected them very appreciably. It is not known as yet how universal this phenomenon is among the bacteria, but our observations on this point are being somewhat extended.

8. *Certain Biochemical Reactions Produced by the Streptococci.* G. J. Hucker, New York Agricultural Experiment Station, Geneva, N. Y.

A study was made of a large number of representative strains of streptococci to find constant and convenient characters by which the species of the genus may be differentiated.   Only authentic and previously named strains of streptococci were investigated and no effort was made to make a floral study of any particular natural habitat of this group.

The streptococci can be divided into two groups on the basis of the type of lactic acid produced.   The low acid producers, which generally prefer levulose to glucose, attack the pentoses, form relatively large amounts of volatile acid, and many times produce carbon dioxide, all form levo-lactic acid.   The more common types of streptococci all produce dextro-lactic acid.

Many of the strains were found to attack the nitrogenous constituents in milk and produce an increasing amount of water-soluble nitrogen if the culture was allowed to incubate over a long period of time in the presence of calcium carbonate.   Certain of the strains studied, particularly *Streptococcus lactis* Löhnis and related types, produced less than 10 per cent of the total acidity as volatile acid, while *S. kefir* Migula, the aroma types, and certain hemolytic strains produced as high as 40 per cent volatile acid.   The relative amounts of nonvolatile and volatile acid formed remained approximately constant regardless of the total amount of acid produced.

The non-hemolytic strains all produce acetic acid and a volatile acid with a higher distilling constant than acetic, while the hemolytic types form, in addition to acetic acid, a volatile acid with a lower distilling constant than that found for acetic acid.   The distilling constants obtained from the hemolytic strains indicate that varying amounts of formic acid are present.

Constant results were obtained in qualitative acid production from sugars if the conditions of growth were controlled, and it was concluded that fermentations of sugars and other carbon sources were valuable as routine methods in separating the species of streptococci.

The strains producing levo-lactic acid were found to hydrolyze the lactose much faster than it was converted into acid, while the remaining types produced acid as rapidly as the lactose was hydrolyzed.

There is a great need for a usable classification of the streptococci, one which is based upon constant and easily determined characters and there is evidence that the biochemical reactions produced by the streptococci studied may be used as a basis for differentiating the species in this genus.

More detailed information may be found in New York State Agricultural Experiment Station Technical Bulletin No. 143.

9. Further Studies on Certain Aciduric Organisms of Dental and Salivary Origin. TOSHIKI MORISHITA, Laboratory of General Bacteriology, Yale University, New Haven, Conn.

In a previous paper the author briefly discussed one of several types of apparently distinct aciduric organisms found in dental caries and in the saliva of persons having carious teeth. This type may briefly be described as follows. The members of this group grow at 12 to 14°C., coagulate milk, and ferment mannose, trehalose, melezitose, mannitol, sorbitol, and salicin, but do not attack sucrose and raffinose. They are agglutinated only by homologous sera.

The present report deals with a second distinct group or type from the same source. This organism is Gram-positive, occurs in rather slender rods, singly or in chains, and tends to form palisade-like groupings. All agar colonies are solid. The different strains do not as a rule coagulate milk; freshly isolated strains may rarely curdle milk but later lose this property. Raffinose and sucrose are fermented. There is no action on trehalose, melezitose, dextrin, sorbitol, mannitol and salicin. The fermentation of mannose is irregular. All mannose non-fermenters are able to grow at 18°C., while many of the mannose-fermenters fail to develop at this temperature. Neither of these 2 sub-types grew at 12 to 14°C. Both produce a large amount of acid in sugar broths and are able to proliferate or remain viable in very acid media. Growth is markedly inhibited by an alkaline reaction. There are at least 2 immunological groups, as determined by means of the agglutination tests.

Group II may be distinguished from *L. acidophilus* (Moro) as follows: (1) While some strains of this group cross-agglutinate in the lower dilutions of heterologous sera, no cross agglutination is observed in the higher dilutions. The presence of these specific and non-specific agglutinins can be demonstrated by agglutinin absorption tests;

by their action in milk; (3) by fermentation of mannose, trehalose,
:trin and salicin; (4) by their relative sensitiveness to chemical
ctions of media; (5) by growth at lower temperatures. Differences in
rphological and cultural characteristics were also found to be of
.ue in differentiation.

Out of a total of 105 strains of aciduric organisms isolated from dental
:ies and from saliva, 30 strains may be placed, because of their bio-
ical reactions, in the dual group reported in this paper. When
nsidered from an immunological viewpoint, this number is increased
39.

Forty-two out of 105 strains fall into the group reported in the
ithor's previous paper. Scattered representatives of a few other
·oups are also found among the remaining strains.

Not more than 7 of the 105 isolations bear a close resemblance to
. *acidophilus* (Moro). The more infrequent types are perhaps more
r less transient, and from the standpoint of possible relationship to
ental caries are of little or no real significance.

*O. A Contribution to the Classification of Microörganisms Treating the
Orders Eubacteriales and Actinomycetales.* ERNST PRIBRAM,
Loyola University School of Medicine, Chicago, Ill.

I. Discussion of general rules for a systematic classification:

1. Characteristics of the higher ranks should contain only the common
:haracteristics, i.e. those characteristics which they have in common with
ιll subordinated groups (orders, families, tribes), not the characteristics
ɔf some of them. Example: Class Schizomycetes: "Undifferentiated
unicellular microörganisms which multiply by cell fission." It is not
necessary to add: "some of them are spore formers." All spore-
forming bacilli are included, because they all multiply by fission also.

2. Characteristics of subordinated groups include of course all char-
acteristics of the higher rank to which they belong and the character-
istics of this higher rank ought not be repeated.

3. The fundamental principle of characteristics, as proliferation,
metabolism, morphology, biological properties should be uniform for all
the coördinated members of one group (class, order, family, tribe).—It
is accepted as a general botanical rule, that the form of reproduction is
of primary importance as far as characteristics are concerned. The
general metabolic properties as for instance direct assimilation of
elements might be superior to morphological characteristics and these
latter again superior to biological properties such as fermentation, pig-
ment production and pathogenicity or toxin production.

4. In a classification one should consider, that there are transitional forms between all species, genera, families, orders and even classes. Example: Spirochetales between Protozoa and Schizomycetes, Actinomycetales between Schizomycetes and Eumycetes. There are therefore characteristics of both groups present in one transitional form. It would be advisable to qualify these groups as *connecting links* and others as *standard groups*. Connecting links have usually species, which vary with greater ease than standard forms (transitional forms). The variation of the connecting links may be due to one or more properties.

5. When classifying fermenting microörganisms according to their power to ferment carbohydrates we should keep in mind that not all the carbohydrates are equivalent. Special studies will be necessary for different families. The property, to ferment lactose for instance, which can be used as one of the main characteristics of the *Escherichia* genus, does not mean simply a difference between the fermentative power of this genus and the other coördinated genera, as *Salmonella*, *Eberthella* etc., but signifies, that the genus *Escherichia* is saprophytic and prefers milk, while the other genera are either parasitic or adapted to other organic products. There are similar findings among different saccharomycetes, e. g., lactase which is a rare ferment of yeasts, has been found in those forms, which are isolated from milk (*Sacch. kefir.*).

6. The reaction with the Gram stain should be widely recognized as a principle of classification, because it very often runs parallel with other important properties as for instance spore formation or anaerobic growth. The Gram technique is not difficult and generally yields good results.

II. In accordance with these principles an attempted classification on the basis of the classification and nomenclature of the Society of American Bacteriologists will be discussed and submitted to this Society. A few names used for subdivisions and some connecting links which have not been approved by the Committee of the Society may be considered as tentative and further discussion on them is invited.

The following families and genera will be discussed with special regard to their position in a systematic classification:

Family Nitrobacteriaceae (completely).
Family Spirillaceae with regard to connecting links.
Family Bacteriaceae. Discussion and classification of all tribes and genera.
Family Bacilleae, remarks on some species.
Family Actinomycetaceae. All tribes and genera.
Family Mycobacteriaceae. All tribes and genera.

11. A Convenient Case for Sterilizing and Storing Micropipettes. WILLIAM H. WRIGHT, University of Wisconsin, Madison, Wisconsin.

The ease with which the delicate tips of micropipettes may be broken during handling and sterilization makes necessary a safe and convenient method of holding them until they are used. The older vertical types of cases for handling micropipettes have the disadvantage that the tips of the pipettes are always upward, and in placing them in such cases as well as when they are taken out they are very liable to be broken or contaminated.

A case of new design has been perfected, and is arranged so that any pipette can be removed at will without molesting the others or running any risk of breakage. In this type of case the pipettes lie parallel to each other in a horizontal row, each one being held in place by an individual spring. Any pipette may be removed at will by one movement of the hand.

12. The Growing of Pure Cultures from Single Cells of Non-Spore Forming Bacteria. WILLIAM H. WRIGHT AND HISASHI NAKAJIMA, University of Wisconsin, Madison, Wisconsin.

A modified cultural technique is described in which the accessory to the Chambers' apparatus, as described by Wright and McCoy, is used to good advantage for both isolating and culturing successfully single cells of *E. coli* and *Pneumococcus*, Type I.

The use of test tube cultures has been abandoned in favor of hanging-drop cultures in special hanging-drop slides with very deep wells. A modified technique is used, in which a small droplet containing a single cell is deposited near the edge of a larger drop of sterile liquid medium on a sterile cover glass over the moist chamber on the stage of the microscope.

By manipulating the tip of a sterile micropipette so that the side of the larger drop near the small droplet is, for an instant only, pulled toward the smaller drop, the two drops are caused to become confluent, and the single cell may be seen to float away into the larger drop.

This technique is more sure than any yet devised and at no time in the procedure is the isolated single cell out of sight of the observer. In the previous method where the tip of the micropipette was broken off in a tube of sterile liquid medium there was always the uncertainty of what had become of the single cell and also a slight chance for air contamination.

During the isolation of over 600 single cell cultures of *E. coli* and

Pneumococcus, Type I, with this improved technique, not a single contamination has occurred. In every case where the single cells transferred to the larger drops did not grow the drops remained sterile for several days until the cultures were discontinued.

Cultures of single cells of non-spore forming bacteria appear to be affected by the following conditions.

1. The relation of the amount of culture medium, (size of culture drop), to the volume of the single cell.   2. Composition of the culture solution from a qualitative point of view.   3. Density of the medium used for the drop cultures.   4. The age of the culture from which the single cells are taken for the drop cultures. Cells give more cultures which grow, when they are removed from cultures in the logarithmic stage of development. This has to be determined by making preliminary trials and counts of the number of cells developing in the medium used and under the conditions used for the drop cultures.

Using this procedure 46 per cent of the single cell cultures of *E. coli* and about 20 per cent of the single cell cultures of *Pneumococcus,* Type I, have been grown.

13. *The Eijkman Fermentation Test as an Aid in the Detection of Fecal Organisms in Water.* LABAN W. LEITER.[1] Presented by WILLIAM W. FORD, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md.

In 1904 Eijkman reported that *Bacillus coli* from man and warm-blooded animals has the ability to ferment glucose broth, with acid and gas production, when incubated at 46°C. and that the contamination of water by fecal organisms can be detected by this method of examination. It has been found, in applying this test to various waters in Baltimore, that it is selective for *Bacillus coli* and that other organisms in water are inhibited or destroyed. It is complete in twenty-four hours. It yields members of the aerogenes-cloacae group infrequently, in marked contrast to the results of Standard Methods. The great majority of the strains of *Bacillus coli* from man and warm-blooded animals are able to ferment glucose broth when grown at 46°C. and the test correlates well with indol production, non-utilization of Koser's sodium citrate and uric acid media. Many strains of bacteria giving the reactions of *Bacillus coli* from feces of cold-blooded animals ferment glucose

[1] Deceased.

broth at 37°C. but fail to grow in glucose broth at 46°C., fail to produce indol, and utilize sodium citrate and the nitrogen in uric acid medium. The Eijkman test may prove to be superior to Standard Methods in the examination of water, in that it eliminates many strains of organisms from cold-blooded animals which react like *Bacillus coli*, but are probably of no significance when found in water. Further studies of the Eijkman reaction are needed.

14. *Hastening Gas Appearance in Presumptive Coli Tests.* JEAN BROADHURST, Teachers College, Columbia University, New York City.

Gas formation may be seen nine to sixteen hours earlier in swimming pool water, polluted well water, etc. by centrifuging the water, and inoculating the usual lactose fermentation tubes with the bottom cubic centimeter in the centrifuged tube. Less difference was found with small samples of water, 10 cc.; with 50 cc., however, the differences were quite consistent, and occasionally, were as great as twenty-four hours in favor of the centrifuged tubes, where gas sometimes appeared in eight hours. Centrifuging was carried on for one hour, full speed, International Equipment Co., centrifuge Size 1, Type C.

15. *The Favorable Rôle of Cysteine in the Cultivation of Anaerobic Organisms.* GEORGE VALLEY, Laboratory of General Bacteriology, Yale University New Haven, Conn.

In their studies concerning the relationship of *B. sporogenes* (*Cl. sporogenes*) to oxygen, Quastel and Stephenson, employing casein digest broth, found that cysteine hydrochloride induced growth of *C. sporogenes* and *Cl. tetanomorphus* in an open tube. In the experiments described here it was deemed of interest to ascertain if other anaerobes would respond similarly. Nutrient broth enriched with cysteine-hydrochloride (0.1 to 0.2 per cent) yielded growth in an open culture tube with the following anaerobes: *Cl. tyrosinogenes*, *Cl. sporogenes*, *Cl. tertium*, *Cl. flabelliferum*, *Cl. chauvei*, *Cl. oedematiens*, *Cl. tetanoides*, *Cl. botulinum* (4 strains), *Cl. welchii* (2 strains), *Cl. tetani* (2 strains), *Cl. histolyticum*, *Cl. sphenoides*, *Cl. bifermentans*, and *Cl. centrosporogenes*. Growth was not obtained with *Cl. putrificum* in an open culture tube; when, however, a small inverted tube (Durham's fermentation tube) was enclosed, growth appeared in the inner tube in from twenty-four to thirty-six hours. The cysteine broth was also employed in carbo-

hydrate fermentation studies with the above-mentioned anaerobes. Results concordant with those of previous investigators were obtained showing that the fermentative and other properties of an organism grown with the aid of cysteine remained unaltered, no matter how luxuriant the growth might be. It is necessary that the cysteine broth be freshly made in order to obtain the desired results.

Methylene blue in high dilution (1:100,000) was completely reduced when added to 5 or 10 cc. of cysteine broth. Cysteine-hydrochloride in the broth medium establishes and maintains a reduced condition sufficient to initiate growth of anaerobic bacteria in an open tube. In an agar medium containing cysteine-hydrochloride (0.15 to 0.2 per cent) reducing conditions were maintained for a considerable length of time, as was shown by methylene blue decolorization. In the plain or glucose agar the indicator was immediately oxidized upon autoclaving, as was shown by the blue color formed throughout the column of agar in the tube. There was, however, a slight reduction at the bottom of the agar column after standing for eighteen hours at room temperature. Evidently auto-oxidation-reduction changes in the medium brought about this subsequent reduction. With cysteine agar the penetration of oxygen was slow, being quite effectively prevented by the reducing agent present. In eighteen hours a blue layer 8 mm. deep developed at the surface; after two days' exposure to ordinary air the blue color was restored to the depth of 20 mm. On liquefying the agar for plating, the blue color was discharged.

Open air incubation with cysteine agar did not yield surface growth on slants excepting with *Cl. tertium*, which showed a small amount of development. There was, however, growth in the condensation water, and also behind the agar, between the glass and the medium. No growth occurred on plates incubated aerobically, the plate pouring manipulation permitting oxygen to penetrate sufficiently to restore the blue color. However, in anaerobic jars, evacuated, refilled 3 times with CO_2, and finally sealed under reduced pressure (25 to 30 mm. of Hg), colony growth was luxuriant with *Cl. tetani* on cysteine-containing agar plates. On the other hand, meager development was obtained on ordinary, cysteine-free glucose agar, and none on plain agar held in the same jars during the eighteen-hour incubation period.

It appears evident that cysteine serves as an excellent reducing agent in the medium and thus establishes favorable conditions for the development of anaerobic organisms.

16. A Modification of McLeod's Anaerobic Plate. P. A. TETRAULT AND
     ELIZABETH McCOY, University of Wisconsin, Madison, Wis.

A simple and yet efficient anaerobic plate may be made as follows:
Two sterile Petri plates of exactly the same size are used. Into
one is poured an agar, gelatin, or silica gel medium inoculated with the
organism to be isolated. This is allowed to solidify. Into the second
Petri plate, pyrogallic acid and sodium or potassium hydroxide are
placed. The part of the first Petri plate containing the solidified
medium is inverted over the half containing the pyrogallic acid-
hydroxide mixture. The two halves are sealed together by means of
heavy gummed paper tape. The tape is moistened and wrapped
around the plate three or four times. The seal is perfect if the Petri
dishes are well chosen.

The amount of pyrogallic acid and hydroxide used varies with condi-
tions under which the organisms are incubated. For a twenty-four to
forty-eight hour incubation the maximum amount of pyrogallic acid-
hydroxide mixture may be used. Using van Riemsdijk's proportions,
10 cc. of 20 per cent NaOH is first placed in the plate and to this is
added 3 cc. of 44 per cent pyrogallic acid. This is sufficient to absorb
the oxygen from 400 cc. of air, twice the amount needed for the volume
of the two Petri dishes. For the longer incubation, from five to twenty-
five days, or at high temperature like 55°C. a weaker mixture must be
used as the hydroxide functions as a dehydrating agent and dries the
agar.

The advantages of the method are: simplicity, no special apparatus
needed, large surface of pyrogallic mixture exposed, process of fishing
out colonies simplified, development of colonies may be watched.

The disadvantages are: pyrogallic mixture may be splashed onto the
agar, perfect seal may not be obtained if plates are not carefully selected.

*17. Quantitative Aspects of the Metabolism of Anaerobes. IV. The Nature
     of the Volatile Acid Produced by C. histolyticum.* W. S. STURGES,
     L. B. PARSONS AND E. T. DRAKE, Laboratory of the Cudahy
     Packing Company, Omaha, Nebraska.

Three strains, carefully identified as pure cultures of *C. histolyticum*,
were grown on a variety of sugar-free media. Fifty-nine Duclaux
volatile acid determinations are presented. They conclusively demon-
strate that this species differs from all anaerobes previously studied by
producing from protein a single volatile acid instead of mixtures of

volatile acids. This one pure volatile acid is produced regardless of protein substrate or age of culture.

18. *Strain Variations in C. bifermentans.* W. S. STURGES AND E. T. DRAKE, Laboratory of the Cudahy Packing Company, Omaha, Nebraska.

Twenty-one strains belonging to the bifermentans-centrosporogenes group (including 4 authentic strains each of *Cl. bifermentans* and *Cl. centrosporogenes*) are compared with each other and with *Cl. sporogenes* as to morphology and certain phases of cultural and biochemical behavior. Differences between *Cl. sporogenes* and the bifermentans-centrosporogenes group are greater than the differences between the individual members of the group. The data suggest that other criteria than motility or ease with which motility may be demonstrated should be used if this group is to be subdivided.

19. *The Identity of Bacillus Sordellii and Clostridium oedematoides.* IVAN C. HALL, University of Colorado School of Medicine, Denver, Colo.; ERWIN JUNGHERR, University of Montana, Bozeman, Mont.; AND MARION REINHARDT RYMER, Highland Hospital, Rochester, N. Y.

During a detailed study of 11 cases of human gaseous gangrene and malignant oedema in South America, Sordelli in 1923 recorded 2 strains of a new species of pathogenic obligately anaerobic bacillus, to which, because of their morphologic and cultural resemblance to *B. sporogenes* and their pathogenic resemblance to *B. oedematiens* (B. Novyi), he gave the name *"B. oedematis sporogenes."* The principal properties ascribed to these 2 strains by Sordelli were confirmed, with the exception of pathogenicity in one of them, by Hall and Scott in 1927, who proposed the binomial *"Bacillus Sordellii"* to supplant the invalid and confusing trinomial first suggested by Sordelli. The pathogenic strain was dissociated by Hall and Scott into 2 strains differing slightly in certain minor characters.

Just a few days before Hall and Scott sent their paper to press, Meleney, Humphreys and Carp described several strains of a supposedly new species of pathogenic anaerobe isolated by them from a fatal case of post-operative infection and from catgut in New York, to which, on account of the resemblance of lesions in the patient and in experimental animals to those of *B. oedematiens* (B. Novyi), and following Bergey's nomenclature, they gave the name *"Clostridium oedematoides."*

The properties ascribed to *Clostridium oedematoides* so closely resembled those of *B. Sordellii* that Hall and Scott suggested the strong possibility of their identity. Humphrey and Meleney have since affirmed it and their findings are here confirmed by detailed comparative morphologic and cultural studies of the South American strains and of the New York strains and by cross protection toxin-antitoxin tests in guinea pigs.

All of the 6 strains available are Gram-positive, obligately anaerobic, slightly motile rods forming subterminal or central clostridial spores. Coagulated albumins and gelatin are liquefied and iron brain medium is blackened. Tyrosin crystals appear in suitable media. One of the South American strains is non-pathogenic but morphologically and culturally identical with the other South American and New York strains. Five of the strains are highly pathogenic for rabbits and guinea pigs, producing lesions similar to those of *B. Novyi*, i.e. marked oedema, with slight congestion, and little or no emphysema, through the action of a true exotoxin. Antitoxic sera were produced in rabbits by the South American strains which protected against both intoxication and infection by the New York strains and vice versa.

In view of the doubtful validity of the genus "Clostridium" the attitude is taken that *"Bacillus Sordellii"* is the first valid binomial applied to this species.

20. Some Factors Influencing the Heat Resistance of Bacterial Spores.   O.
      B. WILLIAMS, Botany Department, University of Texas, Austin,
      Texas.

When cultured and tested under certain standard conditions *B. subtilis* yielded spores of uniform maximum resistance. Pronounced deviations from this basic maximum resistance could be produced by changing the nutritive substrate. Spores of enhanced resistance were produced in peptone water to which had been added either salts of magnesium, phosphates, carbohydrates or certain organic acids. Cultivation in isoelectric gelatin, in various vegetable infusion media or in casein digest medium likewise resulted in the formation of very resistant spores. The temperature of incubation was found to be of some significance, the higher temperatures resulting in spores of increased resistance. The initial reaction of the medium was without influence on the spore produced.

No correlation was apparent between either extent of growth or of sporulation and the resistance of the spore produced.

Attempts to produce spores in any appreciable quantity in synthetic media prepared from highly purified chemicals were not successful.

21. Progress in Standardization of Biological Stains. H. J. CONN, New York Agricultural Experiment Station, Geneva, N. Y.

There are now 38 stains which have been put on the certification basis. There has been a noticeable improvement in the stain supply of recent years as shown by the fact that in 1923 45 per cent of the samples submitted were refused certification, in 1928 only 3 per cent. At the present time steps are under way to secure coöperation with the Federal Specifications Board in drawing up government specifications for stains in harmony with those of the Stain Commission.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

1. Strain Variation in the Root Nodule Bacteria of Clover, Rhizobium trifolii. I. L. BALDWIN AND E. B. FRED, University of Wisconsin, Madison, Wis.

Eighteen strains of *Rh. trifolii* have been tested for their efficiency in aiding plant growth when in association with the host plant. Three of the strains were originally isolated from *Trifolium pratense,* two from *Tr. incarnatum,* one from *Tr. repens,* two from *Tr. suaveolens,* two from *Tr. arvense,* two from *Tr. procumbens,* and six were secured from other investigators or were commercial cultures where the original source of the isolation was unknown. Since their acquisition in this laboratory they have been carried continuously on a yeast water mannitol agar (transfers made every four to six weeks) and tested repeatedly for purity and ability to form nodules on *Trifolium* sp. One strain was secured in 1917, four in 1921, three in 1922, two in 1926, and eight in 1927. With the strains secured from other investigators or commercial cultures the absolute time since isolation from the host plant is unknown.

All tests were made in the greenhouse under controlled conditions. Plants were grown in sterilized glacial sand and watered with distilled water. Mineral nutrients without nitrogen were supplied with a modified Crone's solution. The seed was treated with hot water to kill any nodule bacteria and inoculations were made from young agar slants of the cultures. Plant cultures were always carried in triplicate with frequent controls. All cultures were tested for their effectiveness in aiding plant growth of *Trifolium pratense* and *Tr. repens* two or more times and many have been tested for their effect on other species of clover.

Considerable variation was exhibited with respect to the benefit derived by the host plant from the bacteria. Three of the strains were essentially parasitic in that the growth of the host plant was not in-

creased by the association. Two of these strains were recent isolations, 1927, from *Tr. arvense*, growing on a clover sick soil, while one was isolated in 1921 from *Tr. pratense*. A more or less gradual variation was exhibited between the poorest and the best strains. Under the conditions of these experiments more nodules were formed by the poor strains than by the good strains. The nodules, however, were smaller and were distributed over the entire root system. With the good strains fewer, but larger nodules occurred, and these were located at the upper portion of the root system.

The growth conditions determine to a certain extent the relative value of the strains. Plants grown during the winter with a short day and often little direct sunlight gave less difference between the good and poor strains than plants under conditions more favorable to photosynthesis. Under conditions of low carbohydrate metabolism the need for nitrogen is less and apparently some of the poorer strains are able to supply nitrogen compounds as rapidly as they can be utilized by the host.

2. Relation Between Electrophoresis and Certain Characteristics of the Root Nodule Bacteria. FRANZ ZUCKER, (introduced by E. B. FRED), College of Agriculture, University of Wisconsin, Madison, Wis.

In studying the electrophoretic behavior of the nodule bacteria, 71 cultures have been tested; 31 strains of *Rhizobium meliloti;* 18 of *Rhizobium trifolii;* and 22 of *Rhizobium leguminosarum.* Five day old cultures grown on mannitol yeast water agar with excess calcium carbonate were used. The velocity was determined by Falk's method as given in the Journal of Bacteriology, 15, 367–450, 1928.

The results up to the present show that a certain relationship exists between acid production and electrophoretic velocity. In general the acid formers show a higher velocity than the non-acid producers, with a dividing point at about 4.2 micra per second.

Usually the higher the velocity the "poorer" the cultures are with respect to their effect on the growth of the higher plant. The difference between these good and poor cultures is not as well defined as that between acid-forming and non-acid forming cultures. The lowest velocity was about 2.0 micra per second and the highest was about 7.2 micra per second as measured by the method of Falk. The influence of the treatment of the cultures with respect to the velocity has been studied.

3. Nitrite Production by Some Strains of Cowpea and Soybean Organisms. LEWIS T. LEONARD, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

The ability of certain strains of legume nodule bacteria to produce nitrite in media containing nitrogen compounds, has been demonstrated by some investigators; while others have failed to detect this physiological tendency. Employing principally soil extract and bean extract liquid media with various nitrates and other compounds as sources of nitrogen and sucrose, glucose, dextrin and mannitol as sources of carbohydrate, studies have been made on the production of nitrite on some members of the cowpea group of organisms and on some strains of soybean bacteria including both the high and low nitrogen-fixing types of Wright's designation. As a rule, but not without exceptions, with the cultures used in this experiment, the low nitrogen-fixing strains produced greater amounts of nitrite under given conditions than either the cowpea strains or the high nitrogen-fixing strains of the soybean. In some cases nitrite was apparently produced by the low nitrogen-fixing strains from nitrogen compounds other than nitrate, while some cultures of the other 2 strains did not cause a perceptible formation of nitrite in any of the media employed.

4. Isolation of a Bacteriolytic Principle from the Root Nodules of Red Clover. E. R. HITCHNER, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis., and University of Maine, Orono, Maine.

An agent has been isolated from the root nodules of red clover, which produces lysis of a strain of red clover bacteria isolated from the same nodules. The lytic agent has been carried through 25 serial transfers and filtrations with its homologous organism without any loss in activity. The agent is specific for its own homologous strain and fails to produce lysis of stock strains of red clover organisms. Lysis of a twenty-four hour broth culture is complete in two days. Upon continued incubation of the lysed culture, secondary growth develops. Bacteria, isolated from such secondary growth, resist the lytic action of the agent.

Plate counts made from broth cultures undergoing lysis, showed a progressive decrease in bacterial numbers until the period of secondary growth. At no time was there a complete disappearance of the bacteria. Attempts to produce lysis on solid media by streaking the sensitive organisms and the agent on the surface of plates have failed. Sub-

surface colonies developing in the presence of the agent, however, exhibited a moth-eaten appearance and characteristic lytic areas.

Both the sensitive strain and the resistant strain exhibited all the characteristics of the red clover bacteria and produced nodules on red clover. Both the sensitive and resistant strains have been carried through one plant passage and the organisms reisolated have proved true to original type.

5. *Non-Reciprocal Interchangeability of the Wood's Clover and Garden Bean Nodule Bacteria.* O. H. Sears and F. M. Clark, Agronomy Department, Illinois University, Urbana, Ill.

Detailed studies have confirmed previous observations that the nodule bacteria from legumes in each of 11 cross-inoculation groups do not produce nodules upon Wood's Clover (*Dalea alopecuroides*).

Pure cultures of Wood's Clover nodule bacteria produce nodules in abundance upon the garden bean (*Phaseolus vulgaris*). Pure cultures of organisms isolated from the garden bean nodules infect the dalea plant, only in case the bean inoculation was produced by dalea nodule bacteria.

When judged by nitrogen fixation, the garden bean culture was more effective upon the garden bean plant than the dalea culture, even though each culture produced abundant nodule development.

6. *The Effect of Plant Passage on Certain Strains of the Root Nodule Bacteria.* O. N. Allen and I. L. Baldwin, University of Wisconsin, Madison, Wis.

In the course of the present study, root nodule bacteria of alfalfa *Rhizobium meliloti*, 2 strains, red clover *Rhizobium trifolii*, 3 strains, pea *Rhizobium leguminosarum*, 3 strains, and soybean *Rhizobium japonicum*, 2 strains, were carried through repeated passages in the host plant to study the changes induced in the microörganisms with respect to their ability to benefit the host plants. Pure cultures of Rhizobia were used which had been carried in the laboratory on a yeast water mannitol medium for a number of years without detectable changes in their characteristics. After each plant passage new inoculations were made from a suspension of a crushed nodule. With each species 4 plant passages were secured with two or more strains.

Work was conducted in the greenhouse under controlled conditions and every effort was made to prevent contamination. The seeds were

sterilized in hot water and 1:500 solution of mercuric chloride and inoculated with the organisms immediately before being planted. Triplicate pots of each treatment were carried with frequent controls. In many cases the inocula were secured from nodules of plants grown in cotton-stoppered glass bottles. In measuring the effectiveness of the organism to improve plant growth, the plants were always grown in open pots of sterilized sand. Sterilized distilled water was used to water the plants and the mineral salts necessary for plant growth were made available in sterilized Crone's solution.

In considering the effectiveness or efficiency of the nodule organisms with respect to their ability to aid the growth of the host plants, two factors must be taken into consideration.

(1) The parasitizing effect of the organism on the host, and

(2) The ability of the organism to supply fixed nitrogen compounds to the host plants. Thus the efficiency of any particular organism is a resultant of these two opposing factors and the effects of plant passage on any strain must be studied from both angles.

With certain strains of the organisms the efficiency was increased by plant passage, while with other strains it was decreased. In general each plant passage, within the limits of this investigation, increased the effectiveness of the poorer strains of the organism. The luxuriance of growth of the host plant was increased; there was a gradual increase in the dry weights of the plants and fewer but larger nodules were formed, which were located on or near the upper portion of the tap root. Pure cultures of the better strains of root nodule bacteria showed the reverse. Apparently this decrease in efficiency was due to an increased parasitizing effect rather than a loss of nitrogen fixing ability.

Additional study to see whether these induced characteristics are temporary or permanent under the usual conditions of laboratory maintenance are now in progress.

7. *The Use of Asparagus Extract in Media for the Cultivation of Legume Nodule Bacteria.* W. R. CARROLL, University of Florida, Gainesville, Florida.

A water extract of asparagus has been used as a medium for the cultivation of legume nodule bacteria with good results. The extract compares favorably with yeast water as an accelerator. Mannitol agar containing a small amount of asparagus extract serves well as test medium for *B. radiobacter* contamination.

8. The Gum Produced by the Root Nodule Bacteria of the Leguminosae.
   E. W. HOPKINS, W. H. PETERSON AND E. B. FRED, University of
   Wisconsin, Madison, Wis.

The work reported last year has been extended to include the gum of
the root nodule bacteria. Three strains of heavy gum producing root
nodule bacteria were used for the study. *Rhizobium trifolii, Rhizobium
leguminosarum,* and *Rhizobium meliloti* were grown in pure culture in
synthetic media, the bacteria removed by centrifuging and the gum
precipitated with acetone. It was dried to constant weight and ground
to an impalpable powder. The analyses made included moisture, ash,
uronic acid and pentosans. The uronic acid was determined by the
method of Ling and Nanji, and the pentosans by the method of Young-
burg. The uronic acid varied between 4.1 and 25.3 per cent, the lower
values being obtained when no calcium salts were present in the media.
The pentosan content varied between 4.3 and 16.4 per cent, the lower
values again being obtained when no calcium salts were present in the
media.

Glucose had previously been identified as a product of hydrolysis of
the gum of *Rhizobium meliloti.* Osazones were prepared from the
sugars resulting from the hydrolysis of the gums of *Rhizobium legumino-
sarum,* and *Rhizobium trifolii.* These osazones had melting points
identical with the value of glucosazone, (204°C.). The fermentation of
the hydrolyzed sugar by galactose and non-galactose fermenting yeasts
gave identical fermentations. This was additional evidence that the
sugar was glucose. A fermentation of the hydrolyzed gum by arabinose
and xylose fermenting strains of lactic acid bacteria failed to show the
presence of a pentose.

*9. The Cultural Differentiation of B. radiobacter (Beij.) and Closely
   Related Organisms.* H. E. SAGEN, WILLIAM H. WRIGHT AND
   A. J. RICKER, University of Wisconsin, Madison, Wisconsin.

The marked similarity of the cultural and morphological charac-
teristics of *B. radiobacter* (Beij.), *Bact. tumefaciens* (Smith and Town.)
and many of the legume root nodule bacteria, as well as the occurrence
of all of them in the soil, has been the cause of considerable confusion
when their separation, by cultural means, has been attempted.

Cultural studies of three groups of these organisms, using single cell
cultures of *Bact. tumefaciens* as a basis of comparison, have revealed
differences in respect to: (1) motility; (2) morphology; (3) absorp-

tion of dyes; (4) reduction of nitrates; (5) serological properties; (6) growth in litmus milk; and (7) growth on glycerophosphate media.

Of these differences, the growth on glycerophosphate media is the most characteristic. In this medium with manitol as the source of carbon, radiobacter strains produced abundant growth along with a conspicuous brown halo development around streaks on agar plates. This was accompanied by a whitish precipitate in the agar near the streak. *Bact. tumefaciens* when grown on the same medium produced no brown halo or precipitate.

The radiobacter cultures used did not absorb Congo red while all of the typical crowngall cultures did. The closely related organisms of crowngall which more frequently cause hairyroot in plants, did not absorb Congo red to any marked degree and refused to grow on glycerophosphate media.

In litmus milk cultures of several weeks incubation at 28°C. the hairyroot organisms produced enough acid to turn the litmus and it was often reduced with coagulation of the casein of the milk. None of the radiobacter or crowngall cultures changed the reaction of the milk and it remained a characteristic grayish brown color with a conspicuous serum zone.

Many attempts were made to get the cultures of the typical crowngall organism of single cell origin to show true motility in several liquid culture media but the movement was always observed to be Brownian. The radiobacter strains were the most actively motile and often showed longer cells than the crowngall or hairyroot organisms. The latter strains were demonstrated to have one polar flagellum often attached at a corner of the cell. Flagella have not been demonstrated on the typical crowngall cultures of single cell origin.

Serological studies of many strains of the organisms in all three groups showed no inter-agglutination relation of the groups when tested by both the tube agglutination and short methods. The true crowngall strains were homologous in the same way as the hairyroot and radiobacter strains, there being no splitting up of any of the strains within a group into subgroups or any tendency to cross agglutinate.

10. *Use of the Microscope in Studying the Activities of Bacteria in Soil.*
     H. J. Conn, New York Agricultural Experiment Station, Geneva, N. Y.

The microscopic method for studying bacteria in soil was first proposed in 1917. It was intended at the time as a method of checking up

other procedures for counting bacteria in soil and as a means of determining the morphological types of microörganisms present. Recently Winogradski has employed the method (with slight changes in technic) for observing the kinds of organisms stimulated by addition of various ingredients to soil. The writer in following the line of investigation suggested by Winogradski's work has found another use of the method which promises to be fully as important as those for which it has previously been employed—namely for studying the food requirements of bacteria in soil.

It has been recognized for some time that the behavior of organisms in soil and in laboratory media may be very different. Without question it is more important to know their behavior in soil, but the difficulty of studying their physiology in soil has kept investigators from making much progress in this line. The microscopic method, however, offers a means of studying them. A soil may be selected in which bacteria (or at least some particular organism under investigation) do not grow. Various nutrient materials may be added to different lots of soil and each lot sterilized in test tubes. Then the various tubes may be inoculated with an organism to be studied, and its ability to grow may be learned by microscopic examination. By using this method, interesting information has already been obtained about one previously unknown but very abundant organism in soil, and further data are beng collected about others.

11. Preliminary Bacteriological Studies on Two Acid Soils. NATHAN R. SMITH AND HARRY HUMFELD, U. S. Department of Agriculture, Bureau of Chemistry and Soils, Washington, D. C.

The soils used in these studies were a Leonardtown clay loam, pH 4.6 and a Collington fine sandy loam pH 5.2. Enough of the uncultivated soil to fill eight sections was brought into the greenhouse and placed in benches. The treatments for each soil were as follows: first series—control; limestone only; green rye only; green rye and limestone; second series—control; limestone only; green vetch only; green vetch and limestone. Soil samples were taken before treatment, 2, 4, 7, 14, 21, 35, and 49 days after treatment, and brought into the laboratory for analysis.

The numbers of fungi and actinomyces in both soils remained very constant throughout the experiment regardless of treatment. The pH of the soil with rye green manure fluctuated only slightly, whereas in the case of vetch green manuring there was a tendency for the soil to become

slightly more acid. The amount of nitric nitrogen and the total counts were greatly affected by the treatments. There was a sharp rise in the total counts on the second and fourth days followed by a sharp decline where a green manure with lime was added. This was followed by a gradual rise. In the case of green manure without lime, there was an initial increase in the Collington soil followed by a decrease. However, in the more acid Leonardtown soil there was no initial increase and the numbers remained quite constant although the nitrates increased from 10 to 80 ppm. As a rule, in the early stages of decomposition, nitrates tended to rise with the total counts and after the 7 day sampling, to vary inversely with the total counts.

Small Gram-negative rods were responsible for the greater part of the increases in total counts noted above. The Gram-positive bacteria increased considerably during the first few days after the turning under of the organic matter; they belonged largely to the coccoid and pseudodiphtheroid groups.

The outstanding fact of these experiments is the lack of increase in the numbers of fungi in these acid soils when green rye or vetch is turned under 4 to 5 inches in a greenhouse bench, and the rapid increase in numbers of bacteria on the second and fourth day after treatment.

12. A Practical Method for Determining the Number of Clostridium butyricum Colonies in Acid Soil. HAROLD W. BATCHELOR AND IRVIN H. CURIE, Ohio Agricultural Experiment Station, Wooster, Ohio.

The method is essentially a modification of the Winogradski silicic acid plate method for determining the number of *Azotobacter* colonies in soil. The same inorganic nutrients as recommended by him are used but the calcium carbonate is increased to 2 grams per liter. One per cent glucose is substituted for mannitol and 1.5 per cent Bacto-Difco agar is substituted for the silicic acid.

Seventy-five cubic centimeters of the nutrient agar are pipetted into aluminum Petri dishes having a diameter of 20 cm. and allowed to stand for four to five hours. One-half gram of a soil separate which passes a 20 mesh sieve but does not pass a 40 mesh sieve is distributed uniformly over the solidified agar and allowed to stand in contact with it until the particles are moistened. 100 cc. of the nutrient agar at a temperature of 75 to 80°C. are then pipetted over the particles and allowed to solidify. The plates are incubated at 28°C. for six days. The gas produced through the fermentation of the glucose by the anaerobic bacteria results in the formation of gas bubbles and fractures in the agar around

the soil particles. These fractures are counted with the aid of a brilliant light directed on the surface of the plate. The colonies may be stained by pouring 15 cc. of Lugol's solution over the plate and allowing it to diffuse through the agar. The older colonies containing the typical clostridium forms are stained a deep blue, and the younger colonies a reddish-brown.

The method may be used to determine the presence of both *Azotobacter* and *Clostridium* colonies on the same soil particle by using a double incubation-double pour method. The soil is distributed over the first layer and incubated four days. The second layer of agar is then poured and allowed to solidify. The *Azotobacter* colonies are then counted and identified with a dot of black india ink on the surface of the agar. The plates are then incubated a second time for 6 days and the fractures counted with the aid of red ink.

Data obtained by the method are presented. Limitations of the method and precautions to be observed are discussed.

13. Some Influences of the Development of Higher Plants upon the Abundance and Activities of Microörganisms in the Soil. R. L. STARKEY, Department of Soil Chemistry and Microbiology, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Some of the influences of the development of higher plants upon the microörganisms of the soil have been observed by periodically determining the abundance of certain groups of the organisms and measuring the rates of certain soil processes. In general, the results indicate that plant development brings about a greater development of microörganisms of all kinds about plant roots. The increase is slight in the early stages of root growth, becomes greatest as plants approach maturity and become less pronounced subsequent to death of the plants. The influences of plant development are greatest upon the *Radiobacter* group of bacteria but also are striking upon the bacterial population as a whole. The influences upon actinomyces are not very pronounced. Measurements of the abundance of filamentous fungi as determined by plate counts show no significant consistent influence of plant growth upon their prevalence. The abundance of nitrogen fixing bacteria was not noticeably affected by plant development as measured by growth from dilutions of the soils inoculated into glucose solution. The carbon dioxide evolved from soils obtained from the rhizosphere was greater than that from fallow soils. The greatest production of carbon dioxide accompanied advanced plant development and early degenera-

tion. Nitrification of the soil's own nitrogen was somewhat greater in the planted soils with the advancement of plant growth. In a similar way, the greater the extent of plant development the greater was the influence of the planted soils on accelerating nitrification of added ammonia-nitrogen. In the planted soils there was a greater tendency for consumption of ammonia and nitrate nitrogen suggesting the presence of appreciable amounts of available organic matter of low nitrogen content.

Different plants affected the abundance of organisms and their activities differently but, wherever plant growth was appreciable, marked effects were apparent. The legumes studied did not show any more pronounced effects than some of the non-legumes. The influences of rape, corn and mangel beets were greater than those of beans, sweet clover, table beets, oats or potatoes.

14. Biochemical Aspects of the Acetone Butyl Alcohol Fermentation of Corn Meal. W. H. PETERSON, E. B. FRED AND H. R. STILES, University of Wisconsin, Madison, Wis.

Development of bacteria, degradation of carbohydrates and proteins, and formation of products were determined simultaneously on a single large culture (40 liter culture in a 50 liter Pyrex flask). The maximum number of bacteria (1 to 2 billion per cubic centimeter) is reached in about 30 hours. The increase in numbers of bacteria is accompanied by a rise in the titratable acidity and closely followed by the production of solvents (acetone, butyl alcohol, and ethyl alcohol). A decrease in the number of bacteria and fall in acidity occur simultaneously with the maximum rate of solvent production. All of the solvents are produced simultaneously, but some evidence was obtained that periods of high acidity are characterized by increased acetone and decreased ethyl alcohol production.

The starch content of the medium decreases progressively throughout the fermentation. Reducing sugar reaches a maximum at the end of forty hours and then decreases rapidly. The sugar has been identified as glucose.

Hydrolysis of protein is a concurrent change with acid and solvent production. About two-thirds of the total nitrogen is made soluble. This consists of protein, proteoses, peptones, and amino acids.

The total gas produced during the fermentation consists of about 60 per cent (by volume) of CO_2 and 40 per cent of H_2. During the first three hours it is largely H_2 but after fifteen hours the percentage of CO_2 is greater than that of H_2.

15. The Acceleration of the Alcoholic Fermentation of Cane Molasses by the Use of Vegetable Carbons and Other Inert Substances. WM. L. OWEN AND W. P. DENSON, Department of Bacteriology, Louisiana Experiment Station, Baton Rouge, La.

The alcoholic fermentation of molasses solutions can be greatly accelerated by the addition of small amounts of vegetable carbons. The accelerating effects of these substances while quite pronounced in solutions of ordinary density, is especially so in those of higher density. This acceleration appears to be partly due to the more rapid liberation of CO_2 in the presence of the carbons, but the increased rate of the reduction of acetaldehyde on the carbon surfaces seems to play an important part, as does also the stimulating effect of the carbons upon the yeast themselves. The latter effect is partly to be attributed to the adsorption of toxic substances from the medium. The minute amount of these accelerating substances that is required to produce the stimulating effect upon fermentation suggests a catalytic action, and the fact that substances carrying positive charges are more active in this capacity than negatively charged colloids indicate that the nature of the charge determines to a large extent the value of these substances as accelerators of fermentation. Since the fermentation of some of the hexose sugars is accelerated to a greater extent than others by the presence of carbons an interaction between these substances and the different sugars is indicated. A prior filtration of the molasses solutions through carbons tends to prevent any acceleration of the fermentation of the former when treated subsequently with the latter. The accelerative action of these substances seems greater under conditions where the H-ion concentration is below that required for the optimum rate of fermentation.

16. Changes of the Endpoint of Fermentation by Very Large Seeding. OTTO RAHN, Bacteriology Laboratory, Cornell University, Ithaca, N. Y.

The endpoint of a fermentation, i.e. the maximum amount of products formed in a certain medium, is ordinarily quite constant. It can be increased to a certain extent by increasing the number of acting organisms above the number normally developing in the medium.

By adding large amounts of yeast to a sugar solution very rich in protein, the final amount of alcohol could be increased from the normal 12.0 to 13.9 per cent; in the same way, the amount of CO_2 formed increased from 11.0 to 12.6 per cent of the weight of the medium. This

was accomplished by adding as much as 10 grams of yeast to 100 cc. of medium.

Some streptococci in milk are known to be favored by addition of peptone while others are not. In the first case, the streptococci grow to larger numbers, and produce considerable more titrated acid. A strain of *Str. bovis*, produces 0.73 per cent of lactic acid in milk, and 1.00 per cent in milk plus 1 per cent peptone. This increase is too large to be accounted for by the buffer action of the added peptone. It was not always possible, to establish in this case the same principle as with yeast. The cells from 3000 cc. of a lactose broth culture of the same streptococcus, removed by supercentrifuge and added to 200 cc. of fresh lactose broth, produced the same amount of acid as a normal culture. In milk cultures, large cell quantities gave higher titrated acidity as well as higher [H] ion concentration.

17. Nuts as a Possible Source of Escherichia coli Found in Candy. JOHN WEINZIRL, University of Washington, Seattle, Wash. (Presented by RACHEL E. HOFFSTADT.)

Examinations of 600 samples of nuts of 7 different kinds were made for the presence of *E. coli*, the purpose being to determine whether nuts might be a source of contamination with this organism when incorporated into candy. The samples were secured from a wholesale manufacturer of candy, and retail shops. *E. coli* was found to be present on 0.5 to 2 per cent of the samples, a percentage slightly lower than that found on candy. These results do not indicate that nuts are an important source of the *E. coli* found on candy; rather, they seem to show that, like candy, they are contaminated by the hands of the vendors.

18. Discoloration of Halibut. F. C. HARRISON AND W. SADLER, McGill University, Montreal, Canada.

Over 50 million pounds of halibut are landed yearly on the Pacific Coast. Many of these fish are discolored on the white or belly side. The color is a greenish-yellow. Microscopic examinations of the discolored slime showed numerous bacilli, mostly gram negative, also cocci gram positive and negative. Isolation revealed the presence in large numbers of *Pseudomonas fluorescens*. Pure cultures of the organism caused the typical discoloration on clean fish. The organism was found in large numbers in the artificial ice used for packing the fish in

the boats. Ice all obtained from upland surface waters. This organism also present in large numbers in bilge water (150 million per cubic centimeter) on bins, hold, landing net, etc.

Halibut fresh from the sea, and before being pulled into boat, had no *Ps. fluorescens* on skin or in slime but did have numerous other organisms present. Sea water obtained at depth of 30 to 50 fathoms contained no *Ps. fluorescens* but bacteria present in small numbers. Over 100 isolations of *Ps. fluorescens* were made from as many different boats and various strains studied. Organism grew well at temperatures of 1.5 to 5°C.

19. *Organisms Causing Spoilage of Tomato Products.* CARL S. PEDERSON, New York Agricultural Experiment Station, Geneva, N. Y.

An examination of 83 samples of various spoiled tomato products from various sources and the isolation of the causative organisms has shown that the spoilage of such products is usually caused by certain species of lactic acid producing bacteria. Only 5 yeast cultures and 4 cultures of sporeformers have been isolated. All other cultures have been identified as belonging to one of the following species, *Lactobacillus lycopersici* Mickle, *Bacterium gayoni* Müller-Thurgau and Osterwalder, *Lactobacillus pentoaceticus* Fred and Peterson, *Bacterium mannitopoeum* Müller-Thurgau and Osterwalder, the so-called *Bacillus pleofructi* Savage and Hunwicke or *Lactobacillus plantarum* (Orla-Jensen) Bergey.

Each of the above species has distinct morphological and cultural characteristics by which they can be distinguished from one another. Morphologically all are rods except *Bacillus pleofructi* which ordinarily is a coccus but lengthens at times into rods. It is not a spore-former so that the genus name assigned to it is unsuitable. It is related to the genera *Leuconostoc* van Tiegham and *Betacoccus* Orla-Jensen. The thermal death point of the entire group is relatively low and they are usually killed in the ordinary canning process.

The 5 gas producing species form mannitol from fructose as well as smaller amounts of alcohol, lactic acid, and carbon doxide. Lactic acid and acetic acid are the major end products from the fermentation of pentoses. Lactic and acetic acids, carbon dioxide and alcohol are produced from glucose.

A detailed report will appear as a bulletin from the New York Agricultural Experiment Station.

20. ·The Scope and Findings of Field Work During the Canning Season of 1928. E. J. CAMERON, Research Laboratories, National Canners Association, Washington, D. C.

In 1926 and 1927 studies were made to determine causes leading to specific outbreaks of thermophilic spoilage in canned peas and corn. This work led to the establishment of approximate quantitative standards for the three groups of thermophilic spore-bearing organisms most often concerned in spoilage of non-acid products. Included in this category are (1) the flat sour bacteria causing spoilage through the production of acid without gas, (2) thermophilic anaerobes causing spoilage through the production of both acid and gas, other than H_2S, (3) the so-called sulfide spoilage bacteria (*Cl. nigrificans*, Werkman and Weaver), also thermophilic anaerobes, except in this instance the gas produced is hydrogen sulfide.

From the results of tests on samples representing various stages in the canning process, it was possible to state whether a so-called spoilage hazard existed at the time of sampling, and by a series of such tests to state whether the situation was such as to point to the possibility of future difficulty.

With a view to applying such knowledge as existed on as wide a scale as would be practicable, a large motor truck was equipped as a field laboratory. Culturing operations, incubation and culture readings were done on the truck. The preparation of media, wet and dry sterilizaton and dish washing was done by means of portable equipment which could be carried with the truck and installed in an appropriate part of the cannery where the laboratory for the time would be located. To facilitate matters, basic media were prepared and canned in the Washington Laboratory in advance of the season.

By means of this mobile outfit a field crew of five men were able to collect and examine samples from approximately 125 canning factories in Wisconsin, Minnesota, Iowa and Nebraska. This work was done by establishing the truck laboratory in canneries which were central in the various canning sections and samples were collected from outlying canneries by automobile.

Earlier findings were confirmed. Sugar appears as the outstanding source of thermophilic spoilage bacteria to account for the initial infection of canning equipment and may also at times constitute a direct spoilage hazard. The raw product is an entirely secondary source of spoilage organisms. Wooden tanks used for heating non-acid liquids

may become seeded and infect the canned product. In general, any part of the canning equipment subjected to heat may be involved.

During the course of this work, spoilage hazards were found to be extremely rare. It was found, however, that certain elements of equipment rather often permitted the establishment of thermophilic contamination. Among these should be mentioned the blanching equipment in the canning of peas and pre-cooking apparatus used in canning corn.

21. Growth of Cl. Botulinum in "Waterless-Processed" String Beans.
  LAWRENCE H. JAMES, Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Vegetables canned by the "waterless process" method are not immersed in a liquid, as water or brine, but are filled into the containers, the air removed by a vacuum pump or replaced by steam, the containers sealed and processed. Cans of "waterless processed" vegetables contain no liquid phase in which bacterial contamination might readily spread throughout the container. One bean, infected with *Cl. botulinum* spores, was placed in the center of the top layer of a series of cans of string beans packed by "waterless processing." After incubation the toxicity of the beans at the top, middle, and bottom levels was tested by guinea-pig feeding. The spread of *Cl. botulinum* through string beans so canned was found to be as complete as in vegetables canned in brine. The spread of the organism under such conditions was also demonstrated progressively along a row of adjacent beans placed in a petri dish and incubated under anaerobic conditions. Toxic cultures were isolated from all the beans of the row. The pieces of string beans were also toxic. The entire contents of a can of "waterless processed" string beans may become toxic from the presence of a single infected bean.

22. Studies on Lactic Acid Streptococci. II. On Streptococcus lactis L. and its relation to the fecal streptococci. KARL J. DEMETER, Forschungsanstalt für Milchwirtschaft in Weihenstephan, Germany.

One hundred and six streptococci from milk and fecal sources (man, calf, and insects) were examined. The results of Ayers and his co-workers on *Str. lactis* could be confirmed almost in every sense. This holds also for the fecal streptococci, which gave the same percentage

concerning fermentation of certain carbohydrates. The "order of availability" is the same for the lactic and fecal streptococci. Of the fecal streptococci, about 75 per cent conform to Dible's central type of the enterococcus and its three variants. This is true also with the lactic streptococci. The percentage of Ayers' Var. A (production of gas and ammonia from peptone) and B is the same for the fecal strains as for the lactic strains.

The fermentation of mannite(Andrewes and Horder), of arabinose (Orla-Jensen), blackening of aesculin broth (Meyer and Schonfeld), and the ability to sour milk at 50°C. (Orla-Jensen) do not differentiate the fecal from the lactic streptococci. The litmus milk reaction is of no value either, because the majority of those fecal streptoocci, which at first did not give a typical reaction, did so after being kept in artificial medium (milk) for months or years. This shows clearly that the *Str. lactis* originally may be derived from the intestines and that it still is originating from there. There is no regularity regarding the formation of volatile acids within the lactic strains nor within the fecal strains.

We do not hesitate to say that from the data obtained there is no real difference between the *Str. lactis* and *Str. faecalis* (Enterococcus) and that the name *"Str. lactis"* may be used only to define a local form of the *Str. faecalis*, more or less adapted to the milk medium.

23. Relationships of the Various Acid-Proteolytic Cocci. G. J. HUCKER, New York Agricultural Experiment Station, Geneva, N. Y.

Twenty-five strains of acid-proteolytic cocci were secured from various laboratories and studied to determine the relationships of the types which have been described, some of which have been stated to play an important part in both the normal and abnormal changes in ripening cheese and other dairy products.

It was found that there are two distinct types of acid-proteolytic cocci being studied in various laboratories. One type comprised strains which proved to be micrococci. Belonging to this group were *Micrococcus caseolyticus* Evans 1916 (*M. casei* Hucker 1924), *Tetracoccus casei* Orla-Jensen 1919, *T. liquefaciens* Orla-Jensen 1919, as well as *Mammococcus, Enterococcus, Caseococcus,* and *Gastrococcus* described by Gorini 1926.

The remaining types comprised strains of streptococci which included *S. liquefaciens* Orla-Jensen 1919 and many of the acid-proteolytic types studied by Gorini. It is possible that all the gelatin liquefying strep-

tococci belong to one species with *S. liquefaciens* Orla-Jensen 1919 as the central type.

The acid-proteolytic cocci should not be considered from a systematic standpoint as a separate genus. Many of the organisms of this type being studied in bacteriological laboratories are true streptococci, while certain others are cocci identical with well-recognized and common species of the genus *Micrococcus*.

More detailed information may be found in New York State Agricultural Experiment Station Technical Bulletin No. 144.

24. Reduction of Janus Green B in Milk. H. R. THORNTON, Department of Bacteriology, North Dakota Experiment Station, Fargo, North Dakota.

Workers in Europe have recommended the substitution of janus green B for methylene blue in the reduction test in milk. Janus green B reduces in milk to a red or pink compound thought to be a safranin. This reaction is considered irreversible. This compound further reduces to a leuco-base, which reaction is reversible.

Electrometric studies show that both these reactions take place over potential ranges more negative than that at which methylene blue reduces.

In 157 samples of milk from widely scattered sources the janus green reduction times, with one exception, were greater than the methylene blue reduction times. In one sample the reduction times were the same. The differences varied from an average of thirty-five minutes in milks reducing methylene blue in less than one hour to an average of eighty-three minutes in milks reducing methylene blue in six hours or over. This is accounted for by the difference in the potential ranges over which the two dyes reduce, and by the marked poising action of janus green B.

The endpoint of reduction of janus green B in milk is more indefinite than that of methylene blue. This is due to the poising action of the former and to the difficulty ensuing from the mixture of colors, i.e., the blue of the janus green, the red of the reduced compound, and the white of the milk.

The rate of diffusion of atmospheric oxygen into tubes of whole milk is not rapid enough to have a measurable effect upon the reduction times of methylene blue, janus green B, or litmus. Janus green B showed no advantage over methylene blue as an indicator of quality in milk but several disadvantages were observed.

25. The Bacterial Flora of Pasteurized Milk. H. A. HARDING AND A. R. WARD, Mathews Industries, Inc., Detroit, Mich., AND H. G. HARDING, Akron Pure Milk Co., Akron, Ohio.

It is generally agreed that tubercle bacilli are the most heat resistant of any of the pathogenic bacteria which find their way into the milk supply. The studies of Park have shown that milk heavily seeded with tubercle bacilli is rendered innocuous by heating to 142°F. for ten minutes. Commercial milk pasteurization, as it is almost universally carried on, consists of heating the milk to a minimum of 142°F. for a minimum of thirty minutes. This is three times the treatment time required to totally destroy any pathogenic bacteria which may be present. Accordingly, except when infected after the treatment, time pasteurized milk has no pathogenic flora. Early studies of pasteurized milk were conducted mainly upon an agar medium prepared from Witte peptone, Liebig beef extract and shreaded agar, with or without lactose, and with a reaction of 1.5 to the Fuller scale. The bacteria growing upon such agar suffer a marked reduction in numbers during pasteurization. This reduction is not strictly proportional among all types. It is greater among the non-acid forming bacteria with the result that the proportion of these acid forming bacteria is greater in pasteurized milk than in the raw milk before pasteurization. Incident to war conditions, the use of a variety of peptones and of beef extracts in preparing agar media with a wide range of reaction led to the discovery of a thermophilic flora in pasteurized milk. Study of this thermophilic flora has been handicapped by the lack of convenient methods for recognizing the presence and the relative numbers of its representatives. In 1923 Harding and Ward reported that the presence of some representatives of this thermophilic flora could be shown by incubating standard agar plates at 62.5°C. (145°F.). The methylene blue reductase test conducted at 100°F. is being widely used to determine the presence and relative numbers in raw milk of the flora which thrives at 100°F. We have found that the presence and relative numbers of the thermophilic flora in pasteurized milk can likewise be determined by the application of the methylene blue reductase test by carrying out this test at 145°F. (62.5°C.). This same methylene blue test at 145°F. can be used to determine the presence of thermophiles in raw milk. They have been found in all samples of raw milk yet examined, though the numbers present vary widely. Entering the pasteurizing machinery with the raw milk, the thermophilic bacteria attach themselves to the walls of the apparatus in which the milk is held at pasteurizing tempera-

ture and there multiply rapidly. As the pasteurizing process is continued these thermophilic bacteria pass out in increasing numbers with the pasteurized milk. Direct microscopic examination of the pasteurized product shows a steady and rapid increase in this thermophilic flora from approximately 1,000,000 per cubic centimeter early in the process to many millions when the pasteurizing process has been continued for six or more hours. There is no evidence that the presence of this flora has any sanitary significance and except under unusual conditions it does not have any commercial significance.

26. *Thermophilic Spore-Formers Associated with Milk.* PAUL S. PRICKETT, New York Agricultural Experiment Station, Geneva, N. Y.

In studying the problem of unusually high counts from pasteurized milk a total of 480 cultures of thermoduric (heat-resistant) and thermophilic (heat-loving) organisms have been secured from a variety of sources. This total includes seven types of organisms which are listed in the order of their importance based on the number of times each type was isolated in this study: Spore-forming rods, streptococci, non-spore-forming rods, micrococci, *Actinomyces,* sarcinae, and a yeast.

The cultures of facultative and obligate thermophilic spore-formers isolated in this study were divided into 10 groups or species based on their morphological, cultural and physiological characteristics. The names assigned to the 10 groups or species are as follows: *Bacillus subtilis* Cohn, as described by Gottheil and Ford (not a true thermophile) *B. terminalis* Migula var. *thermophilus* Prickett; *B. michaelisii* Prickett (probable synonym, *B. ;thermophilus-aquatilis-liquefaciens* Michaelis); *B. calidus* Blau; *B. thermoalimentophilus* Weinzirl; *B. aerothermophilus* Weinzirl; *B. thermoliquefaciens* Bergey; *B. nondiastaticus* Bergey; *B. calidolactis* Hussong and Hammer; and *B. kaustophilus* Prickett.

The results of this study and the results of previous studies by other workers indicate that the majority of the thermophilic spore-formers are characterized by being aerobic, and by having terminally located ellipsoidal to cylindrical spores as well as by their thermal relations. Of these 10 species, only 2 were found to have centrally located spores, namely: *Bacillus nondiastaticus* Bergey, and *B. subtilis* Cohn. Under special conditions these spore-forming bacteria may occur in pasteurized milk in enormous numbers.

A complete report of this work has just been published as Tech. Bull. No. 147 of the New York Agricultural Experiment Station, Geneva, N. Y.

27. The Significance of Thermophilic Spore-forming Bacteria in Pasteurized Milk. ROBERT S. BREED, PAUL S. PRICKETT AND M. W. YALE, New York Agricultural Experiment Station, Geneva, N. Y.

Investigations by the authors have shown that truly thermophilic bacteria rather frequently find conditions favorable for growth during the pasteurization of milk, particularly in large plants where the pasteurizing equipment is kept continuously in operation for from three to twelve hours or even longer. The significance of the presence of these organisms is largely dependent upon their origin, upon the conditions under which they grow in the milk and upon the effect of their activity in milk.

Organisms of the types present in pasteurized milk are almost universally distributed in soil. They are also universally present in hay, grain, silage and manure, being associated with the heating of these materials. As they usually form spores, it is natural to find these spores present in the dust and similar materials of the cow stable. Even the cleanest milk may occasionally contain small numbers of thermophilic bacteria.

The only dairy utensil that offers favorable conditions for these bacteria seems to be the milk can as these frequently stand exposed to the sun for a sufficient length of time to be heated to temperatures favorable for the growth of thermophilic bacteria. Tests have shown that these organisms may occur in milk cans.

It does not seem probable that especially favorable conditions for growth occur normally before the milk reaches the pasteurizing plant. During pasteurization, however, there are many opportunities offered for their growth as for example in (*a*) foam which may remain in the pasteurizing vat for hours; (*b*) in milk cooked onto the metal pipes or other equipment where the water or steam used in heating the milk is at a temperature above 150–152°F.; (*c*) dead ends which cause eddies in the current of hot milk; (*d*) gaskets, pitted metal surfaces and other rough places which offer conditions favorable for the development of these bacteria.

During pasteurization, delays caused by lack of milk at the receiving platform, lack of bottles at the bottler or other things cause milk to be held longer than thirty minutes at pasteurizing temperature and so give a chance for the growth of the thermophilic bacteria. Another rather common practice in milk plants is to repasteurize milk returned from delivery wagons or milk drained from the heater, holder or bottler at the end of the day's run. All of this milk is apt to contain large numbers of

thermophiles and serves to inoculate the fresh milk with thermophilic bacteria.

The chief effect of the presence of millions of the thermophilic bacteria per cubic centimeter is to cause a change in the reaction of the milk which is usually indicated by an increased acidity, although some of these thermophilic types are liquefiers and alkali producers. A number of large milk plants report that the growth of these bacteria affects the flavor of the milk. Both of these characteristics make their presence highly undesirable from the commercial standpoint. As their presence in large numbers is due largely to favorable temperatures, these bacteria do not have an intimate connection with unsanitary practices. It is self-evident also that obligate thermophilic bacteria cannot be pathogenic in the ordinary sense as they do not grow at temperatures as cold as those found in the human body. Likewise there is no evidence that indicates that facultative thermophilic bacteria are pathogenic.

Detailed reports of this investigation have appeared or will appear as bulletins of the New York Agricultural Experiment Station.

28. The Effect of Heat Produced by an Alternating Electric Current on Tubercle Bacilli in Milk. C .M. CARPENTER, Diagnostic Laboratory, New York State Veterinary College, Cornell University, Ithaca, N. Y.

Raw and pasteurized milk heavily inoculated with human and bovine tubercle bacilli was subjected to a 220-volt 60-cycle alternating current by pumping the milk between two carbon electrodes. The milk passing between the electrodes completed the circuit and, by its resistance to the current, raised the temperatures of the milk to 150°F., 155°F., and 160°F. Guinea pigs injected with milk treated in this manner showed no evidence of tuberculosis when the milk was heated to 155°F. and 160°F. Some of the guinea pigs injected with milk treated at 150°F. developed typical lesions of the disease.

29. The Bacterial Count of Ice Cream Held at Freezing Temperatures. JOHN WEINZIRL AND ALICE E. GERDEMAN, Department of Bacteriology, University of Washington, Seattle, Washington. (Presented by Rachel E. Hoffstadt.)

Thirty-four samples or bricks of ice cream from two producers were kept at temperatures below zero centigrade and examined for numbers of bacteria per gram on alternate days. Twelve samples were held at minus 3°C., 12 at minus 6°C., and 9 at minus 10°C. The results indi-

cate that storage of ice cream at minus 10°C. or above, does not prevent all bacterial multiplication or the increase of the bacterial count. The results furnish at least a partial explanation of the increased numbers of bacteria found in retailers' samples as compared with those from producers.

30. Contaminated Water as a Source of Surface Flavor in Pasteurized Creamery Butter. DONALD BETHUNE SHUTT, Laboratory of Bacteriology, Ontario Agricultural College, Guelph, Canada.

Surface flavor butter is almost unknown in butter made in city creameries, where the water supplies are known to be pure, but it would appear that this flavor is quite prevalent in creameries in the rural districts, especially after prolonged periods of wet weather.

All suspected water supplies have been found to be contaminated with *Pseudomonas fluorescens.*

Sterile butter when inoculated with this organism and incubated at 25°C. for 28 days developed typical surface flavor. Investigations in the field and laboratory proved that this flavor could be controlled by: substitution of pure water for contaminated water for butter washing, subjecting contaminated water to a temperature of 190°F. for 10 minutes, neutralizing the cream to not less than 0.35 per cent acid.

31. Outbreak of Mold in a Cheese Factory. D. H. DUNHAM, University of Wisconsin, Madison, Wis.

A factory manufacturing a soft bacterial ripened cheese known in the trade as "Liederkranz" was losing a considerable portion of its product through the profuse growth of a *Penicillium* thereon. The manufacturing operations had been recently transferred from a factory of rather primitive type to a modern structure designed for the special purpose. The equipment for maintaining the proper temperature and humidity of the curing room and the relation of the various rooms to each other were such as to force the air with its load of mold spores from the curing room into the room, known as the making room, in which the cheeses were kept for a few days following their manufacture. The condition of the cheese in the making room in regard to moisture and salt content was such as to favor the germination of the mold spores. The condition in the curing room was unfavorable for spore germination, but did not inhibit to any marked extent vegetative growth and the production of spores.

The fumigation of the various rooms was impossible without closing

the factory.   The trouble was gradually overcome and finally reduced to a very low level by: (1) A general factory renovation; (2) The isolation of the making room from the curing room; (3) The sterilization of all materials such as boards and racks as they passed from the curing room to the making room to be used once more; (4) The use of a slightly lower temperature in the making room to inhibit spore germination.

IMMUNOLOGY AND COMPARATIVE PATHOLOGY

1. Effect of Oral Administration of Ox-Bile on Appearance of Agglutinins for Esch. coli in Sera of Normal Rabbits.   LA VERNE BARNES, The Bacteriological Laboratories, State College of Washington, Pullman, Wash.

Besredka believes that the oral administration of ox-bile to rabbits renders the intestinal mucosa more pervious to bacterial protein.   The following experiment was devised to test this theory:

Cultures of *Esch. coli* were isolated from the feces of normal rabbits. Gram negative, short rods were in predominance in samples of feces studied.   The agglutination titers of the sera of 5 normal rabbits for *Esch. coli* were determined.   Ox-bile, in doses of 10 cc., was given by mouth to 3 rabbits on alternate days until a total of 80 cc. had been administered.   The agglutination titers were then determined at varying intervals.   The last agglutination test was made approximately 90 days after the last dose of bile.   No change in the agglutination titers was observed.

2. Effect of Oral and Subcutaneous Injections of Pasteurella avicida in Rabbits Vaccinated by the Oral and Intravenous Methods.   LA-VERNE BARNES, The Bacteriological Laboratories, State College of Washington, Pullman, Wash.

Four rabbits were given oral injections of *Past. avicida* vaccine, and 5 others were vaccinated intravenously.   The agglutination titers were determined.   The animals were then given doses of virulent cultures of *Past. avicida* as follows: 2 orally, and 3 intravenously vaccinated rabbits were given the test doses by mouth.   The remaining animals in each group were injected subcutaneously.   Controls were injected for each group.   All 5 of the control rabbits died in less than 24 hours.   Results in the vaccinated animals do not indicate any difference in the degree of protection by the two methods used.   The results have a bearing on the theory of local immunity.

3. A Note on the Use of "Specific Wet Dressings" of Staphylococci According to the Method of Besredka. LaVERNE BARNES, The Bacteriological Laboratories, State College of Washington, Pullman, Wash.

Pure cultures of staphylococci were isolated from boils on the faces of two students. Wet dressings of the filtrates of broth cultures, as suggested by Besredka, were applied on only one side of the face to determine whether a local immunity would develop. Immunity was not developed in 24 hours as claimed by Besredka. Before any observations as to the general effect could be made, the patients had applied the filtrates to the entire face. In both cases definite improvement was reported. No attempt was made to isolate *B. acne* from these cases.

At the present time, vaccines of *Esch. coli* and *Eber. typhi* are being applied cutaneously to determine whether any agglutinin formation will take place. Negative results in this respect have been obtained to date.

4. Immunological Studies of Typhoid Vaccination by Mouth. 1. Agglutinins Formed in Persons Treated Orally with Triple Typhoid Bacterin. RACHEL E. HOFFSTADT AND RANDALL L. THOMPSON, Department of Bacteriology, University of Washington, Seattle, Wash. (Presented by RACHEL E. HOFFSTADT.)

Ninety-three persons were treated orally with liquid triple typhoid bacterin. Tests were made at weekly intervals for 11 weeks and at the end of 5 and 9 months. Only in 1 case was there a systemic response. Of those treated, 83.5 per cent showed agglutinins for typhoid and a lesser number for paratyphoid. Bile prepared individuals showed a higher percentage of agglutinins than non-bile prepared persons. The latent period for the appearance of agglutinins was 3 to 4 weeks. Titers of 600 were reached. No higher dilution was run in the tests. Maximum titers were reached in the fourth to fifth weeks. Duration of agglutinins in a fair proportion of individuals was demonstrated at the end of 5 and 9 months. Previous treatment with the subcutaneous method or a history of typhoid fever had no effect except to raise the titers.

5. Immunological Studies of Typhoid Vaccination by Mouth. 2. Complement Fixations and Precipitin Reactions in Persons Treated Orally with Bile and Triple Typhoid Bacterin. RACHEL E. HOFFSTADT AND RANDALL L. THOMPSON, Department of Bacteriology,

University of Washington, Seattle, Wash.   (Presented by RACHEL
E. HOFFSTADT).

Thirty-nine persons were treated orally with liquid typhoid bacterin.
Eighty-eight per cent of all persons showed positive fixation and pre-
cipitins.   Complement fixation tests and precipitin tests were made at
weekly intervals over a period of 11 weeks and at the end of 5 months.
Bile seems to have no effect on the production of complement fixations
and precipitins.   Latent period for the appearance of complement fixa-
tions for typhoid is 4 to 5 weeks.   Duration is irregular for both fixa-
tion and precipitins.   Complement fixations are demonstrated after
5 months.   There seems to be a definite relationship between the
appearance of fixations and agglutinins in this group but it corresponds
to that reported for cases of typhoid fever.

6. *Immunological Studies of Typhoid Vaccination by Mouth. 3. Agglu-
    tinins and Complement Fixation Reactions Formed in Persons
    Treated with Typhoid Bacterin in Capsule.*   RACHEL E. HOFF-
    STADT AND CARL E. MARTIN, Department of Bacteriology,
    University of Washington, Seattle, Wash. (Presented by
    RACHEL E. HOFFSTADT.)

Forty-two persons were treated orally with a dry triple typhoid
bacterin in the form of a capsule.   A marked systemic response was
found in 2 persons only.   Eighty-seven per cent of the persons treated
gave positive agglutinins.   Seventy-nine per cent gave positive com-
plement fixation.   The latent period for agglutinins was 3 to 4 weeks,
and for complement fixations, 4 to 5 weeks.   Titers of agglutinins
compared well with those produced by the liquid bacterin.   Persons
in which the bile was given separately from the capsule showed a more
ready response than those who had a capsule in which the bile was
incorporated.

7. *Bacteriophage as a Substitute for the Typhoid Vaccine.*   N. W. LAR-
    KUM, Michigan Department of Health, Lansing, Michigan.

Bacteriophage prepared against *B. typhosus, B. paratyphosus* A and
B has been used for immunization against typhoid fever.   About 1200
inmates of state institutions have been inoculated and the serological
response in about 200 has been studied.   The study has been concerned
with the type of reaction following injection, the agglutination, opsonic
and bactericidal response, the effect of variations in dosage, and the
duration of the antibodies produced as compared with the usual vaccine.
One inoculation of 2.0 cc. of bacteriphage has resulted in the production

of agglutinins to the same titre as produced by the vaccine. The bactericidal power of the blood and the opsonic index of those inoculated with bacteriophage has been higher than those produced by vaccine. After 6 months antibodies were present to a higher titre in those having a single inoculation of bacteriophage than in those having a single inoculation of vaccine, and equal to the antibody titre in those having three inoculations of vaccine. Three inoculations of bacteriophage gave less satisfactory results than did a single inoculation.

8. *The Effect of Specific Sera Upon the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.* GREGORY SHWARTZMAN, Laboratories of Mount Sinai Hospital, New York City.

A phenomenon of local skin reactivity to *B. typhosus* culture filtrate was previously described (J. Exp. Med., xlviii, 1928–247). The reactivity was induced by skin injections of the filtrate. If 24 hours later an intravenous injection of the same filtrate was given to rabbits there appeared an extremely severe hemorrhagic necrosis at the site of previous skin injections. There were found certain features *which considered together* distinguished this phenomenon sharply from the known phenomena of bacterial allergy and the Arthus phenomenon.

Further work is reported on the relation of the specific antisera to the factors which induce the skin reactivity, so called "skin preparatory factors." The neutralization of these factors is easily accomplished by specific sera. In order to apply this phenomenon to practical purposes neutralization experiments were performed on a large number of rabbits with the following results:

It is found that there is a definite variation in the response of different rabbits to the skin preparatory effect of neutralized mixtures. The reactions obtained by injection of the same mixtures into different animals are divided into three categories: completely neutralized (CN) partially neutralized (PN) and non-neutralized (NN). It is also found that the potency of a given serum bears a mathematical ratio to all the types of reactions, namely to both the titer of serum giving complete neutralization in CN and PN rabbits and the number of rabbits belonging to *all the three types*. A scheme is proposed for carrying out accurate quantitative titrations of the protective value of serum (a complete report will appear elsewhere).

Normal and non-related heterologous sera have no neutralizing effect unless natural agglutinins are found for *B. typhosus*. Para A and B sera neutralize these factors in various dilutions. Whether the Para A and B antibodies are of a group character or identical with the *B. typhosus* antibodies remains to be determined.

9. An Improved Technique for the Comparison of Antiseptics by the Yeast Fermentation Method. SARA E. BRANHAM, Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

A simple apparatus has been devised in which carbon dioxide production by fermenting yeasts can be measured with a fair degree of accuracy. By means of this apparatus the principles underlying the yeast fermentation method of comparing the antiseptic efficiency of silver and mercury compounds have been confirmed, put upon a quantitative basis, and shown to be applicable to many other types of disinfectants. In addition to giving a useful means of comparing antiseptics, this device offers an opportunity for the study of the phases of yeast fermentation under the influence of different compounds. The extreme simplicity of this technique commends it for general use.

10. A Comparison of the Hygienic Laboratory Test with the Method Used by the Department of Agriculture for Testing Disinfectants. C. M. BREWER AND GEORGE F. REDDISH, Food, Drug and Insecticide Administration, Department of Agriculture, Washington, D. C.

The advantages of the method for testing disinfectants used by the Department of Agriculture (described by Reddish in previous publications) as compared with the Hygienic Laboratory method are again set forth. Because of the very wide use of compounds chemically related to phenol for disinfecting purposes, members of this group were selected for comparing the two methods of testing. Twenty representative coaltar disinfectants and 10 cresylic acid compounds were used, the *B. typhosus* phenol coefficient being obtained by both methods. The differences obtained by these methods are given. In some cases a higher phenol coefficient is obtained by the Department of Agriculture method and in others the Hygienic Laboratory method gives a higher figure, depending on the composition of the disinfectant. The figures show that the average percentage of variation between the two tests is too small to be of practical significance, seldom being higher than the limits of experimental error.

11. The Antiseptic Action of U. S. P. and N. F. Ointments. GEORGE F. REDDISH AND H. WALES, Food, Drug and Insecticide Administration, Department of Agriculture, Washington, D. C.

U. S. P. and N. F. ointments which are being used for antiseptic purposes were tested for bacteriostatic action by a method previously

described by the senior author. The ointments were streaked on the surface of plain nutrient agar (1.5 per cent agar) inoculated with *Staph. aureus.* Antiseptic action is indicated by a clear zone surrounding the streaked ointment, the remainder of the plate being turbid with the colonies of the test organism. Using this method of testing, it was found that only about one-half the U. S. P. and N. F. ointments so tested are in fact antiseptic.

12. *The Variation of the Phenol Coefficients of Coal Tar Disinfectants with the Use of Different Test Organisms.* BURTON G. PHILBRICK, Skinner, Sherman and Esselen, Inc., Boston, Mass.

For the purposes of this study, 4 samples of coal tar disinfectants having approximate *B. typhosus* phenol coefficients of 2/3, 5/6, 9/10 and 20, respectively, were selected as representative of that class of disinfectants.

The exact phenol coefficients of these samples were then determined by the modified Rideal-Walker method using *B. typhosus* as the test organism with and without the presence of organic matter. The *B. typhosus* phenol coefficient having been established, the phenol coefficients of these same samples were determined using as test organisms *Staph. aureus, B. diphtheriae, Strep. hemolyticus,* and *Pneumococcus.* In these determinations the culture medium was necessarily modified to suit the organism, but the modified Rideal-Walker method was adhered to as to temperature of medication, amount of inoculent, and time of exposure to disinfectant.

The phenol coefficients as determined are given in the following table:

SAMPLE	2/3	5/6	9/10	20
B. typhosus without organic matter..................	3.2	6.5	10.0	19.0
B. typhosus with organic matter.....................	2.9	5.8	9.0	17.0
Staphylococcus aureus without organic matter........	0.8	1.4	2.2	5.0
Staphylococcus aureus with organic matter...........	0.7	1.3	1.8	4.9
B. diphtheriae without organic matter...............	2.3	4.5	7.3	18.0
B. diphtheriae with organic matter..................	1.6	3.5	5.3	14.0
Streptococcus hemolyticus without organic matter....	2.2	4.4	6.7	16.0
Streptococcus hemolyticus with organic matter..	1.7	3.5	5.3	12.0
Pneumococcus without organic matter...............	3.3	6.6	10.0	23.5
Pneumococcus with organic matter..................	3.0	6.1	9.4	17.5

13. Bactericidal Effects of Sunlight as Affected by Glass. THEODORE W. HAUSMANN, Concordia College.

The relative rates of bacterial destruction by sunlight filtered through plate glass and through glass permeable to ultra-violet light (using plates $\frac{3}{16}$ inch in thickness) were determined with *Escherichia coli, Serratia marcescens* and *Staphylococcus aureus* (Reddish strain).

The first tests were made (August to October 15) by exposing pour plates and surface plates containing varying dilutions of each of these organisms to mid-day sunlight (1) without any intervening barrier, (2) through plate glass and (3) through glass permeable to ultra-violet light. (Vita-glass).

For these experiments special cubical boxes were constructed, 20 inches in diameter, with ventilating apertures to prevent condensation of moisture and to control possible differences in temperature (readings being made from 3 thermometers similarly placed in each box). The boxes were placed on adjustable supports to aid in keeping the glass covers and the contained Petri dishes at right angles to the sun's rays. Exposures varied from 30 minutes to 5 hours in the different experiments, varying with the known resistance of the organism and the variations in light intensity. After exposure, the plates were incubated for 2 days (37°C., except for *Serratia,* where 21°C. was used). The following conclusions may be made: (1) *Staphylococcus* was more rapidly affected by light than the other 2 organisms, one-half hour of exposure yielding marked contrasts; with *E. coli,* 1 hour was usually necessary, and even in August 2 hours were needed for marked contrasts with *Serratia marcescens.* (2) There were in all marked differences in colony size as well as in the number of colonies developing. (3) The ratio of the colonies developing may be roughly averaged down to the following proportions: 11, no sun or shade; 8, plate glass; 5, glass permeable to ultra-violet light and 3, direct sunlight.

The second set of tests (October and November) were made on the bacterial content of air in enclosed spaces. These tests offered greater difficulties, because of the frequency of air contaminants (chiefly molds), even when the exposures were made on the roof of a five-story building in the city. These air tests were, at first, made by washing the air exhausted for anaerobic jars in each box and left open during exposure. They were then closed and the exhausted air was washed through a series of water bottles, and the wash water used for plating. The plate-glass box air was higher in bacterial content than that in the other box covered

with glass permeable to ultra-violet light. Later, the boxes were treated with sulphur dioxide, ventilated (wet cotton filtering incoming air) and sprayed with bacterial cultures with atomizers. The air was pumped from these boxes into wash bottles as before, but as yet, practically no growth has been obtained, either because the wet spray reaches and adheres to the box surfaces or because the particles settle down during exposure to the sun.

14. The Use of Sodium Chloride in Culture Media for the Separation of Certain Gram-Positive Cocci from Gram-Negative Bacilli. Justina H. Hill and Edwin C. White, Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Md.

It was found that pH 6.0 sodium chloride agars, with salt concentrations from 2 through 20 per cent exerted marked inhibitory action on the growth of bacilli of the typhoid, paratyphoid, dysentery and colon groups, on *Proteus, Pseudomonas pyocyanea,* diphtheroids and *Bacillus anthracis.* The Gram-positive cocci studied tolerated high salt concentrations, all growing on transfer from 20 per cent sodium chloride agar.

In pH 6.0 broths with salt concentrations from 2 through 25 per cent, the same differential action was observed, although to a lesser degree than on agar.

With cultures of cocci and of bacilli, mixed in different proportions, the salt agars gave a selective growth of the cocci which could sometimes be recovered in pure culture. The use of 6, 8, 10 and 15 per cent salt agars greatly facilitated the isolation of Gram-positive cocci from mixed infections.

15. A Simplified Culture Medium for the Isolation of Neisseria gonorrhoeae. Jas. Gibbard, Department of Pensions and National Health, Ottawa, Canada.

A new culture medium for the isolation and cultivation of *Neisseria gonorrhoeae* is described in detail. The basis of the medium is veal infusion, peptone, starch and agar plus human serum. The source of human serum is the surplus from specimens submitted for the Wassermann test. The medium is very easy to prepare and gives excellent growth of the gonococcus, usually within 15 to 24 hours. In the author's hands, it has proven superior to the media recommended by various workers within the past 10 years. It can be accurately standardized, thus assuring little if any variation between different batches.

16. An Analysis of Six Thousand Consecutive Routine Blood Cultures.
W. G. HEEKS AND L. W. FAMULENER, St. Luke's Hospital, New York City.

In a series of six thousand consecutive blood cultures, (1,024) or 17.06 per cent, showed bacterial growth; of which 27 or 0.45 per cent of the total were considered as contaminants.

Seven hundred and seventy-one individuals yielded positive results, and in 253 instances, the blood cultures were repeated one or more times with positive findings.

The order of occurrence of various organisms appearing 15 or more times, in the series of positive individual cases, is as follows:

	Number of individuals	Per cent of positive cultures
Pneumococcus	214	27.7
B. typhosus	197	25.5
Strep. viridans	93	12.0
Strep. hemolyticus	89	11.5
Staph. aureus	45	5.8
Staph. aureus-hemolyticus	23	2.9
Staph. albus	15	1.9
Staph. albus-hemolyticus	15	1.9

It is of special interest to note the time periods at which the growth of various organisms appeared. The average time for *Streptococcus viridans* was 2.3 days, while *Streptococcus hemolyticus* appeared in 1.6 days; *B. typhosus* averaged 2.0 days; *Staphylococcus aureus* 2.0 days; *Staphylococcus albus* 1.5 days, and *Pneumococcus* (all types), 1.3 days.

17. Method of Securing Blood Serum from Convalescent Patients. F. M. MEADER, Department of Health, City of Detroit.

The value of blood serum from persons who have recovered from various communicable diseases, as a prophylactic agent is well substantiated in the medical literature. The problem of securing such serums in sufficient quantities has been solved by the Detroit Department of Health in the following manner:

The Department decided to secure serum from convalescent cases of measles, for distribution to institutions and doctors wishing to protect young or feeble contacts. The name and address of adults who had measles during the past year was noted. A form letter was sent to each one, offering ten dollars for a sample of blood. A nurse was detailed to arrange with donors who accepted the offer, to come to the laboratory where a doctor would secure the blood. By this means 2,200 doses were

secured. Details regarding method of handling the serum, amount of blood obtained, the cost, and distribution, are given in the paper.

The same method has been used to secure blood serum from persons convalescent from scarlet fever, whooping cough, mumps, chickenpox and poliomyelitis, with as good results. The serum section of our laboratory promises to be a very important service in our Health Department.

18. Seasonal Variation in Antitoxin Content of Human Blood. ROGER
    G. PERKINS, RALPH HEEREN, EMERSON MEGRAIL, AND A. B.
    GROSSMAN, *Department of Hygiene and Bacteriology, School of
    Medicine, Western Reserve University, Cleveland, Ohio.*

A 12-month study of changes in the antitoxin content of the blood in 2 age groups involving 30 individuals, with consideration of possible relations to the seasonal incidence of diphtheria. This paper forms part of a series of studies of seasonal and periodic variations in a group of diseases.

19. Further Studies on the Immunology of the Pneumococcus. H. M.
    POWELL, G. H. A. CLOWES, AND W. A. JAMIESON, *Eli Lilly and
    Company, Indianapolis, Indiana.*

Further work upon the immunology of the pneumococcus has shown that toxic filtrates of this organism can be reliably evaluated by skin tests on different species and immune serums can be prepared which are neutralizing. Such pneumococcus serums can be concentrated. It has been found that standardization of pneumococcus filtrates and serums by mouse lung tests as previously reported in Proceedings of the Society for Experimental Biology and Medicine, 1926, vol. **23**, pp. 331–337, is more or less uncertain and difficult to control. The very high degree of purification of serums reported has been impossible to obtain as judged by skin neutralization tests. The skin test method, however, offers a means by which pneumococcus toxic filtrates and serums can be measured.

20. An Auxiliary Antigen for the Kahn Test. R. L. KAHN, G. LUBIN AND
    E. McDERMOTT, *Michigan Department of Health, Lansing,
    Mich. and University of Michigan Hospital, Ann Arbor, Mich.*

Several years ago one of us described an antigen for use in the "presumptive" Kahn procedure. That antigen, while more sensitive than standard antigen, lacked the property of uniformity in sensitiveness due

to our meager knowledge of the principles of antigen standardization. With a better understanding of these principles, it was possible to prepare and standardize an antigen more sensitive than standard antigen and possessing at the same time a high degree of uniformity. This antigen was named "sensitized" antigen.

Three reagents are necessary in the preparation of sensitized antigen: (1) standard antigen, (2) sensitizing reagent which consists of an alcoholic solution of dried ether extractives obtained from the preparation of standard antigen and (3) an alcoholic solution containing 6 mgm. cholesterol per cc. Sensitizing reagent added to standard antigen up to certain limits, increases the sensitiveness of the final product. When adding cholesterolized alcohol to this product, there is a further tendency for increasing the sensitiveness. By the use of sensitizing reagent and cholesterolized alcohol, standard antigen can thus be increased in sensitiveness to the level of sensitized antigen, by the employment of standard sensitized antigen as a control.

The presumptive procedure with sensitized antigen is markedly more sensitive than the 3-tube Kahn test in treated cases, this increase being as high as 30 per cent. In diagnostic cases the increase in sensitiveness of the presumptive over the regular test depends on the type of syphilis among the patients tested. At the University of Michigan Hospital this increase approximates 2 per cent. With regard to the specificity of the presumptive procedure, no opportunity has presented itself thus far for careful clinical studies. Preliminary studies indicate however that this procedure is specific to a high degree.

21. Studies on the Kahn Reaction with Spinal Fluids. R. L. KAHN, E. McDERMOTT AND G. LUBIN, Michigan Department of Health, Lansing, Mich. and University of Michigan Hospital, Ann Arbor, Mich.

The original Kahn test with spinal fluids was highly specific and conservative in sensitiveness. Special precautions leading to conservative sensitiveness were considered essential because the method of precipitating the globulin of spinal fluid with saturated ammonium sulfate resulted in the presence of about 3 per cent of this salt in the final globulin solution—a concentration which, even in the absence of positive serum or spinal fluid, is sufficient to yield slight precipitates with antigen suspension when the latter is prepared at the usual serum titer. To overcome this difficulty the lipid particles in the antigen suspension were made more soluble by preparing the suspension with a

larger amount of saline than that indicated by the antigen titer for serum tests. Furthermore, only 40 instead of 50 per cent of the saturated ammonium sulfate was used in the separation of the globulins. Both of these precautions tended to reduce the sensitiveness of the test.

It was recently observed that by employing small (7.5 by 1 cm.) instead of large test tubes for mixing the spinal fluid with the ammonium sulfate, and by using a few simple precautions in manipulation, it is possible to remove almost completely the supernatant fluid from the globulin precipitates, thus reducing to a minimum the amount of sulfate in the globulin solution. This technique made practicable a 50 instead of 40 per cent saturation of the spinal fluid with ammonium sulfate, yielding all of the fluid globulins instead of only a fraction of the globulins. It also made practicable the use of an antigen titer similar to that employed in serum tests. In a series of 400 spinal fluid tests run under these conditions, some increase in sensitiveness was observed without encountering false positive reactions.

It was also found that sensitized antigen may be used in the spinal fluid procedure. The reactions obtained in a group of 300 spinal fluids indicate a higher degree of sensitiveness with freedom from false reactions. A still more sensitive spinal fluid reaction is obtained with sensitized antigen when the globulin concentration of these fluids is increased 20 instead of 10 times the concentration in the original fluid. The group of fluids studied is as yet too small to establish the specificity of this method but indications are that this procedure is also specific.

It appears that the Kahn reaction lends itself to the employment of spinal fluid procedures of marked sensitiveness and specificity in the detection of neurosyphilis.

22. Primary Diphtheric Vaginitis in Children. ANNA I. VAN SAUN AND DOROTHY V. FREEMAN, Board of Health, City of Paterson, N. J.

A short resumé is given of the few authentic cases of primary diphtheric vaginitis found in the literature.

The authors report a case of apparently primary diphtheric vaginitis in a child 8 years of age, giving a description of the clinical aspects of the case and the bacteriologic findings.

Cultures taken from the throat of the patient contained no diphtheria bacilli. Those from the nose contained a diphtheria-like bacillus which, on being tested for virulence, gave negative results. Cultures from the vagina contained large numbers of typical diphtheria bacilli

which gave a positive virulence test. The case is thought to have been probably primarily a vaginal infection since nose and throat cultures were consistently negative.

Attention is called to the necessity for release cultures in cases of vaginitis as well as in other forms of diphtheria. The necessity for virulence tests is also stressed when the determination of the original avenue of infection is indicated.

23. *Corynebacterium ulcerans: Its Possible Epidemiological Importance.* RUTH GILBERT, Division of Laboratories and Research, New York State Department of Health, Albany, New York.

The close resemblance of *Corynebacterium ulcerans* in young cultures to the diphtheria bacillus and its coccoid appearance after longer incubation makes animal inoculation necessary for identification. Since most throat cultures receive a morphological examination only, the prevalence of infections due to *C. ulcerans* may be greater than past experience has indicated. It has proved to be the incitant in at least two epidemics in widely separated districts in New York State.

24. *The Influence of Carbon Dioxide on Diphtheria Toxin Production and Preservation.* WAYNE N. PLASTRIDGE AND LEO F. RETTGER, Laboratory of General Bacteriology, Yale University, New Haven, Conn.

In the study of the influence of varying amounts of CO_2 on the diphtheria bacillus and its extracellular toxin both the subcutaneous and intracutaneous methods of toxicity determination were employed. The carbon dioxide and oxygen were supplied to the cultures by an aeration system with which fairly constant amounts of these gases could be maintained.

Aeration of cultures with CO_2-free atmospheres containing from 10 to 50 per cent oxygen resulted in marked irregularity in growth and in the toxin content of *C. diphtheriae* cultures, and permitted an increased rate of destruction of toxin once it was formed. The rate of destruction of toxin in these cultures was directly proportional to the concentration of oxygen in the atmospheres passed over the cultures.

The presence of from 3 to 10 per cent CO_2 in the atmospheres passed over *C. diphtheriae* cultures resulted in increased growth and toxin production. Uniform maximum toxin formation with a given medium was obtained in cultures grown under an atmosphere containing 10 per

cent CO_2 and from 10 to 20 per cent oxygen. No decrease in toxicity occurred in any of these cultures on prolonged incubation.

Growth and toxin production were slightly retarded in cultures aerated with an atmosphere containing 5 per cent oxygen and markedly retarded in cultures aerated with an atmosphere containing only 1.5 per cent oxygen.

Tests conducted for the purpose of determining the mechanism responsible for the preservation of diphtheria toxin in cultures grown under an atmosphere containing from 3 to 10 per cent CO_2 showed the following: (1) *In the absence of added* CO_2, the rate of destruction of toxin by molecular oxygen, within the limits of pH 7.0 to pH 9.0, is inversely proportional to the hydrogen ion concentration of the toxin solution; (2) *In the presence of added* CO_2, the destruction of diphtheria toxin by molecular oxygen is largely or entirely prevented.

Toxic culture filtrates were adjusted to different hydrogen ion concentrations and stored in sealed tubes under nitrogen, CO_2, air, and in *vacuo*. Only the filtrates stored under CO_2 remained highly toxic after 180 days storage at 37°C.

The general conclusion may be drawn from the data presented, that CO_2 plays an important rôle in the growth and toxin production of *C. diphtheriae*, mainly by (1) stimulating growth and toxin formation, (2) controlling the reaction of the culture medium during the growth of *C. diphtheriae*, and (3) preventing the destruction of the toxin once it is formed.

25. Bacteriological Characteristics of Streptococcus epidemicus Isolated During a Septic Sore Throat Epidemic. ELLIOTT S. ROBINSON, Antitoxin and Vaccine Laboratory, Jamaica Plain, Boston, Mass.

The morphological and cultural characteristics of strains of *S. epidemicus* isolated from patients affected during an extensive epidemic of septic sore throat and from the cow whose milk was the probable source of the infection were studied. The strains isolated were alike in being encapsulated hemolytic streptococci growing as moist colonies on milk-blood-agar medium; in being virulent for mice; in producing acid in glucose, lactose, sucrose, and salicin media but not in inulin, mannite, or raffinose; and in being low acid producers (pH 5.3 to 5.7) in glucose broth. In short, the organisms studied were entirely similar to those described by others from previous epidemics of septic sore throat. The course of the natural infection in the cow was also followed for a time.

26. The Origin and Nature of the Epithelial Cell Inclusions in Trachoma and Their Relationship to the Disease. IDA A. BENGSTON, Hygienic Laboratory, U. S. Public Health Service, Washington, D. C.

A microscopical study has been made of film preparations from numerous cases of trachoma with a view to determining the nature of the inclusion body and its relationship to the disease. It appears that the inclusion body originates from a rod-shaped microörganism and that the "elementary bodies" of Lindner are modified forms of the rod-shaped microörganism. It is probable that the lytic substances present in the conjunctival fluids and in the conjunctival cells act on the rod-shaped bacteria reducing them to the small, indefinite pleomorphic forms in which they occur outside the cells and that the inclusion bodies in the epithelial cells are formed as the result of the action of lytic or digestive substances on a group or colony of bacteria contained within the cytoplasm of the cell. Forms resembling the "elementary bodies" and the bipolarly stained "free initial bodies" of Lindner have been produced experimentally by inoculating the conjunctiva of guinea pigs with cultures of certain Gram-negative rod-shaped microörganisms isolated from cases of human trachoma. The study tends to confirm the work of Noguchi who found a certain Gram-negative rod associated with the disease and to disprove the theory of Lindner that the inclusion body originates from an organism which is non-bacterial in nature.

27. Epizootic Lymphadenitis in the Guinea-Pig. EMERSON MEGRAIL AND ROBERT N. HOYT, Department of Hygiene and Bacteriology, Western Reserve School of Medicine, Cleveland, Ohio.

Thirty-five guinea-pigs were examined in an outbreak of epizootic lymphadenitis, commonly known as "lumps." Either a hemolytic or non-hemolytic streptococcus and, in addition, *Staphylococcus albus* were isolated from each animal. Experimentally, both types of the streptococcus are able to produce the disease and the staphylococcus is apparently a secondary invader. Transfer experiments through skin abrasions were successful.

28. Experimental Transmission of Epizootic Encephalitis of Foxes. R. G. GREEN AND N. R. ZIEGLER, Department of Bacteriology and Immunology, University of Minnesota, AND E. T. DEWEY AND J. E. SHILLINGER, Bureau of Biological Survey, Washington, D. C.

Epizootic encephalitis of foxes has been transmitted to well, healthy

foxes by the injection of brain and spinal cord virus obtained from animals dying of the natural disease. The identity of the disease with the artificial infection is shown by the period of infection, the symptoms and pathology produced. The disease has been transmitted by intracranial, cisterna and intramuscular injection. The infection has been transmitted in series from one experimental animal to another.

29. Experimental Tularemia Infection in Birds. R. G. Green and E. M. Wade, Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minnesota, and the Division of Preventable Diseases, Minnesota State Board of Health.

Skin inoculation of the ruffed grouse and the Hungarian partridge with material infective for tularemia, results in a fatal infection, terminating in about 7 days. Upon intramuscular injection a generalized infection usually occurs in the common pigeon. The infection does not result fatally and no symptoms of the disease are evident. *Bact. tularense* appears to be eliminated from the body of the pigeon in about 7 days.

Intramuscular injection of infective material in the ring-necked pheasant results in a generalized invasion with no apparent symptoms of the disease. Recovery from the infection occurs after about 7 days.

30. Chromogenesis in B. salmonicida. David L. Belding and Julia G. Arrowood, Boston University School of Medicine and Evans Memorial Hospital, Boston, Mass.

B. salmonicida, the causative agent of fish "Furunculosis," produces a diffusible brown pigment when grown upon favorable media at room temperature. The pigment is slightly soluble in alcohol, but not in chloroform or ether. Pigment formation may be temporarily inhibited by cultivation under anaerobic conditions, upon acid media, upon media containing certain antiseptics, and at a temperature above 30°C. Chromogenesis is restored when the organism is subcultured under favorable conditions.

A sport strain obtained from a 7-year-old stock culture proved nonchromogenic. Except for pigment formation it was bacteriologically and serologically identical with the chromogenic strain from which it was derived and with strains of *B. salmonicida* from other sources. Efforts to convert the non-chromogenic into a chromogenic strain by various methods of cultivation have so far been unsuccessful.

31. A Preliminary Note on the Cytology of Mycobacterium tuberculosis. GEORGES KNAYSI, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

The young cell of *Mycobacterium tuberculosis* consists of a very thin membrane presenting thickened areas and granular appendages over its internal surface. The cytoplasm is made up of dense, chromatic material and many vacuoles. In the chromatic material are granules of a hyperchromatic substance apparently identical with the substance of the membrane. Those granules vary in size and number. The larger ones are most commonly two in number, one near each pole of the cell. A third granule may be in the middle, or two or more distributed along the cell. Almost invariably, a granule lies contiguous to a vacuole. The substance making up the granules and the membrane is highly elastic and is extremely resistant to chemicals. It was not dissolved, at the end of one week, by glacial acetic acid, 5 per cent sulfuric acid, 5 per cent sodium hydroxide, or chloroform. It takes up strongly iodine, all of the basic dyes tried, and the fat dyes. In taking up dyes, granules and membrane swell. Especially with fat dyes is this effect remarkable when both the membrane and the granules may attain more than twice their normal thickness. This substance is the principal cause of the acid and gram resistance of the organism. The granules, which are very conspicuous in an actively growing culture, must be, because of their great adsorptive power, the foci of intense chemical activities, and they probably play a preponderant rôle in the nutrition of the cell. Two of these granules are usually formed, when the cell is dividing at the contiguous poles of the daughter cells, but their origin and their behavior, as far as are known, do not justify considering them as nuclei as we know nuclei in higher plants and animals.

No plasmodesma were observed between cells, and no wax, neutral fat, or free fatty acids were demonstrated microchemically either within the cell or around it, as is generally believed, in cultures from 5 to 24 hours old.

32. Filtration Experiments with the Granular Form of the Tubercle Bacillus. RALPH R. MELLON AND ELIZABETH L. JOST, Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh, Pa.

For the past 5 years French workers especially have been most active in their attempts to demonstrate a filterable phase of the tubercle bacillus. Despite their asseverations that their contention is proved, the evidence submitted has been quite inadequate in almost every instance.

Progressive disease has not been produced, and they have not been able to isolate the tubercle bacillus from supposed (?) lesions where acid fast bacilli were demonstrable in the smears. Moreover, histological evidence for tubercle has usually been lacking.

In 1926 we reported in a preliminary way definitely positive results, satisfying all the above criteria. But the inoculation conditions, as well as the material itself, were somewhat anomalous and it was desired to repeat the experiment with other similar material and under varying conditions of inoculation.

Our inoculation results have been categorically positive *only* when the inoculation material contained large numbers of what appeared to be acid fast granules. Out of a total of 37 animals, 4 have yielded widespread progressive lesions of the disease. From 2 of these animals the tubercle bacillus in pure culture has been recovered. These 4 positive animals are the result of inoculation of granular material from 2 sources; one, a sputum; the other, a pulmonary miliary tuberculosis.

Although many of the remainder of the 37 animals had enlarged lymph glands with acid fast bacilli, thus corresponding with the bulk of the French work, we do not consider such evidence adequate for the filtrability contention unless supported by the evidence shown for our 4 definitely positive animals.

33. *Permeability of the Respiratory Tract to Old Tuberculin.* PAUL F. CLARK AND (by invitation) WILLIAM S. MIDDLETON AND OTIS M. WILSON, *University of Wisconsin, Madison, Wisconsin.*

Several acute attacks of asthma occurred in a student whose duties exposed him to the vapor of tuberculin in a room where this material was being cleared by means of a Sharpless centrifuge. As the patient gave a negative von Pirquet reaction, we wondered whether so poor an antigen as tuberculin could possibly have been the means of sensitizing this individual through the respiratory tract. We have succeeded in demonstrating that it is easy to sensitize guinea-pigs to such excellent complex antigens as horse serum and such simple proteins as crystallized egg albumen or excelsin by the use of a fine spray in a closed chamber. We have also sensitized guinea-pigs to old tuberculin by this method so that subsequent intravenous injections of minute amounts have resulted in fatal anaphylactic shock.

34. *A Study of Bacillus Calmette-Guerin (B.C.G.). 1. Biological Characteristics, Cultural "Dissociation" and Animal Experimenta-*

tion. S. A. PETROFF, ARNOLD BRANCH AND WILLIAM STEEN-
KEN, JR., Research and Clinical Laboratory, Trudeau Sana-
torium, Trudeau, N. Y.

Prophylactic immunization against tuberculosis has been extensively
carried on in Europe. The method consists of feeding infants with
living avirulent cultures of tubercle bacilli known as *Bacillus Calmette-
Guerin* (B.C.G.). The claims have been that the organism does not
produce progressive tuberculosis and that it possesses remarkable
antigenic properties for rendering humans and animals immune to
tuberculosis. Our studies since 1925 can be summarized as follows:

1. The organism when cultivated on a medium advocated by the
sponsors of this method, only on very rare occasions produces tubercu-
losis in animals terminating in death.

2. After the organism has been dissociated into "R" and "S" colonies,
it was found that the "R" colony produces tubercles and the lesions have
a tendency to heal, while the "S" invariably produces extensive lesions
leading to the death of the animal.

3. The immunity established in guinea pigs with this organism is not
greater than that obtained when animals are injected with heat-killed
tubercle bacilli.

The use of this type of vaccination is not entirely free from the danger
of infection in human beings. More work must be done on a larger
scale, especially in cattle, in order to substantiate or refute the claims of
the authors of this method.

35. The Pathogenicity of the Species of the Genus Brucella for Monkeys.
I. FOREST HUDDLESON, Department of Bacteriology, Michigan
State College, East Lansing, Mich.

Experiments to determine the pathogenicity of strains of each of the
3 species of the genus *Brucella* for monkeys by the oral route reveal that
the porcine species is the most pathogenic. This is true of this particular
species regardless of the source of isolation, (hog, cow, or man).

The true bovine abortus species, regardless of its source, will produce
a mild form of the disease followed by rapid recovery after the ingestion
of a massive dose or repeated small doses over a considerable period of
time.

Small doses of a recently isolated strain of the melitensis species will
produce the disease in monkeys, but old cultures fed daily for a long
period of time fail to produce the disease.

In nearly every case one agar slant of the porcine species has produced the disease in 10 to 15 days without recovery.

These findings are suggestive of the difference in virulence of the species for man.

36. The Significance of Brucella abortus Agglutinins in Human Sera. C. M. CARPENTER, RUTH BOAK, Diagnostic Laboratory, New York State Veterinary College, Cornell University, Ithaca, N. Y., AND O. D. CHAPMAN, Department of Bacteriology, School of Medicine, Syracuse University, Syracuse, N. Y.

A comparison of the results obtained by investigators in various states of the percentages of Wassermann serum samples showing *Br. abortus* agglutinins is reported. A study of 4,050 serum samples collected at Syracuse, N. Y. showed 7.3 per cent to contain *Br. abortus* agglutinins while 2.4 per cent of 955 samples collected from Bellevue Hospital, New York City, were positive.

The blood serum from individuals fed pasteurized milk with a high *Br. abortus* agglutinin titre showed no evidence of the absorption of the antibodies. Examinations of the blood serum of individuals having recovered from undulant fever demonstrated the antibodies to be present in some cases for a period of 2 years after the symptoms had subsided. *Br. abortus* has been isolated from the blood of patients with undulant fever showing no agglutinins as well as from cases with very few agglutinins in their serum. Because of such findings it is impossible to designate the minimum serum titre diagnostic of undulant fever.

37. The Respiration of the Abortus-Melitensis Group. M. H. SOULE, Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.

The respiratory quotients were determined for human, porcine and bovine cultures of *Brucella abortus* and for cultures of *B. melitensis*. The organisms were grown on agar slants in *h*-tubes connected to manometers. Samples of the supernatant gases were removed from the respiratory chambers and analyzed with the modified Henderson-Haldane burette. The CO_2 held in the media (dissolved and fixed) was determined by aeration. From the total amount of O_2 consumed and CO_2 produced the corrected real respiratory quotients were calculated. The rates of growth were followed manometrically. The observed manometric readings were compared with those computed from the analyses of the gases and thus served as a check on the analyses. Manometrically the porcine strains gave the most rapid growth and the

melitensis strains the slowest growth on all of the mediums investigated
The human strains according to their rate of growth were either of the
porcine or bovine type.

On plain infusion agar there were no marked differences in the
corrected real respiratory quotients for the various strains, indicating
similar metabolic activities. The average corrected real respiratory
quotient was 1.056, much higher than the accepted value of 0.81 for
protein substances. This value may be taken as correct for the experi-
mental conditions which prevailed; but it probably does not represent
the actual intracellular respiratory change. Secondary extracellular
changes such as carboxylase action, where CO_2 is formed, could increase
the total yield of CO_2 with a corresponding increase in the respiratory
quotient.

The addition of 2 per cent glucose to the medium favored the growth
of all of the cultures, and exerted a sparing action on the decomposition
of the proteins implying a utilization of glucose. There was a distinct
decrease in the quantities of fixed CO_2 in these experiments, the bovine
strains gave the smallest amounts. The average corrected real respira-
tory quotient on the glucose containing medium was 1.046.

On 5 per cent glycerol agar rich growths were obtained. The average
corrected real respiratory quotient was 0.950, distinctly lower than on
the other two mediums but much higher than the theoretical value of
0.857 for the combustion of glycerol. The smallest amounts of fixed
CO_2 observed were obtained with this medium indicating the utilization
of glycerol for growth energy with a minimum amount of protein
decomposition.

Distilled water suspensions of the organisms were killed when spread
over the surface of beef extract agar in Kitasato plates and subsequently
aerated with CO_2-free air, CO_2-free tank nitrogen ($\pm$ 5 per cent O_2)
or purified tank N_2 (CO_2-free, oxygen free) at the rate of 100 cc. per min.
Luxuriant growths were obtained if a small amount of CO_2 was added to
the aerating gas. These experiments are being continued in an effort to
detect some variation in the gaseous metabolism of these strains.

*38. An Organism Isolated from Three Cases of Bacteremia After Appendi-
citis.* EMIL WEISS, Department of Bacteriology, Loyola Uni-
versity School of Medicine, Chicago, Ill.

The isolated organism shows the following properties: short rods
with round ends, 0.3 to 0.5 micron wide, 1 to 1.5 microns long, occurring
singly, sometimes in pairs. The organism is non-motile, has no flagella,

does not form spores, has no capsule and is Gram-negative. The colonies on gelatin are small, circular and transparent; liquefaction occurs on gelatin stab. The colonies on agar are small, transparent and glistening. The broth shows a slight turbidity, has no pellicle and forms a scant sediment. Litmus milk remains unchanged after several days of incubation. On potato only a scant growth could be obtained. The organism does not produce indol, and does not reduce nitrates. It does not form acid or gas in carbohydrate media. It produces no characteristic odor and is a facultative anaerobe. The optimal temperature for growth is 37°C. It is non-pathogenic for rabbits, guinea pigs, white rats and mice. According to the described properties this organism must be classified in the order Eubacteriales, and genus Alcaligenes. Within this genus it seems to be principally related to the *Alcaligenes abortus* (Bang).

39. The Application of Weiss' Flagella Method for the Staining of Spirochetes. EMIL WEISS, Department of Bacteriology, Loyola University School of Medicine, Chicago, Ill.

The above method gives the following advantages, if applied to the demonstration of spirochetes: the mordant keeps, and does not need to be freshly prepared; the spirochetes appear large and deeply stained; no sediment is noticeable; and when using a combination of a contrasting basic and an acidic dye, the bacteria take the basic dye, the spirochetes the acid dye.

LIFE CYCLES IN BACTERIA[1]

ALICE C. EVANS

Bacteriologist, Hygienic Laboratory, United States Public Health Service, Washington, D. C.

Life cycles are a law of nature. In the highest forms of life the changes are obvious because they are gradual, and the individuals are so large that transformations may be readily followed. On the other hand, in lower forms of life the transformations may be abrupt and therefore difficult to follow. In microscopic organisms the changes are particularly difficult to follow because continuous observation of an individual organism and of its progeny requires great patience and skill. In single-celled organisms the parent cell may be of one form, and all its descendants may be of another form.

. Presumably it was on account of the difficulties in following bacterial changes that the monomorphic theory was adopted. No doubt the investigations of the early bacteriologists were more productive of practical knowledge under the monomorphic theory than would have been possible under a pleomorphic theory, for the monomorphic theory held in check the confusion which would have arisen in the new science if attention had been diverted to the intricate problems of life cycles. No science has a more brilliant decade's record than was made by bacteriology, particularly by medical bacteriology, in the 80's under the monomorphic theory.

Bacteriology cannot progress, however, if original theories are held tenaciously when evidence accumulates against them. Chemists, physicists, geologists and astronomers are continually altering theories to fit newly discovered facts. Bacteriologists must do likewise or their science will lag. A constantly increas-

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[1] Presidential address delivered at the thirtieth annual meeting of the Society of American Bacteriologists, Richmond, Virginia, December 28, 1928.

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ing amount of evidence is accumulating against the theory of monomorphism in bacterial organisms. It is the purpose of this paper to examine this evidence in the light of the general law of life cycles as illustrated by familiar organisms standing higher than bacteria in the scale of life.

A brief survey of the general law of life cycles may be obtained by considering their occurrence in three widely separated groups— (1) vertebrates, (2) insects, (3) fungi and algae. These groups include representatives of the highest, an intermediary and the lowest forms of life, and they include groups from both the animal and the plant kingdoms.

Some bacteriologists have expressed their inability to accept the theory of bacterial pleomorphism because they say that if they accepted pleomorphism, they would also have to accept complete mutation in biologic behavior. When changes of form have been reported, they involved one or more other changes as, for example, changes in habitat or in physiologic behavior, or, in the case of pathogenic bacteria, changes in pathogenicity or in immunologic relationships. The fact that bacteria of a given form may be maintained indefinitely in culture media presents another difficulty. If bacteria undergo life cycles, why is it that certain cultures have been maintained for many years, even for many decades, without a change of form?

It is in order to consider some of these perplexities from the point of view of general biology that the examples of life cycles to be cited are chosen. From them several deductions may be made: (1) Life cycles are a law of nature. (2) Changes in morphology may be abrupt. (3) Changes in morphology are accompanied by changes in biologic behavior. (4) Environment conditions the development of inherited characters. (5) There may be irregularities in the life cycle. (6) The reproduction of a single phase of the cycle may continue relatively indefinitely. (7) Irregularities in life cycles depend on external influences.

The cycle in man, and in most other vertebrates, proceeds in regular order. The differences in the characteristics of the young and the adult are considerable, however, even in man. Suppose that our planet were to be visited by an intelligent being

from Mars, and that the Martian, although of a form entirely different from man, possessed the same kind of mental faculties, and that he had developed his faculties along the same lines as a bacteriologist. If his first view of human beings were limited to two individuals, a mother and her young infant, he would probably think he had found two species, one parasitic upon the other, for the two would not look alike, they would not act alike, one would be twenty times the size of the other, and most important to his bacteriological mind, the two individuals would not eat the same kinds of food. If the Martian would prolong his visit, however, so that he could observe a large number of human beings, he would find every gradation in size and behavior between the extremes of mother and child. He would have to modify his preliminary hypothesis and conclude that the two individuals represent different stages in the life cycle of one species.

Although gradual changes are the rule in vertebrates, sudden changes, called metamorphoses, occur in certain species of that group, as for example, in amphibians and the lamprey. In the frog, metamorphosis occurs at the end of the aquatic or tadpole stage. At this time there is a sudden bursting into function of organs which developed gradually during the previous tadpole life. There are structural changes in the organs of the respiratory and vascular systems, and the paired limbs appear. These changes are required for the terrestrial life of the adult.

In the Hexapoda, or insects, metamorphoses, or sudden changes, are the general rule. Most insects pass through a life cycle of four distinct phases—egg, larva, pupa and adult. In certain species of insects, however, there are notable irregularities in the cycle. A brief review will be given of the life history of the rosy aphid (*Anuraphis roseus*), an insect whose life history exhibits a variety of forms and remarkable irregularities depending on environment. Eggs of the rosy aphid laid on the apple tree in the fall hatch at about the time the buds break in the spring. The aphids hatching from the winter eggs are called stem-mothers. The stem-mother matures in about fifteen days, whereupon she begins producing young at an average of six a day. She lives

from a month to six weeks. Her offspring are of a rosy color and may be either winged or wingless. The production of these forms on the apple continues for several generations. When mature, the winged forms fly to plantains where they produce young throughout the summer months. Most of the aphids produced upon the plantain are wingless, although a few winged ones occur throughout the summer. Generation after generation develops on the plantain. Their color is yellowish green. As autumn approaches winged forms, consisting of both males and gamogenetic females, appear on the plantain. They are able to live and reproduce only upon the apple or closely related species. These fall migrants mate and produce on the apple leaves wingless, orange-yellow females, which complete the cycle by laying the winter eggs.

There are other species of aphids, which have life histories even more strange than that of the apple aphid. In *Aphis rosae*, the plant-louse of the rose, the young may be born by the oviparous or by the viviparous method, and either gamogenetically or parthenogenetically, and they may develop into winged forms or remain wingless. If the food plant is grown in a conservatory with protection against cold, the rose aphid may go on reproducing parthenogenetically without cessation for many years.

Another familiar example from insect life illustrates strikingly how external influences determine the development of one or another form of an organism. The kind of food supplied to the larvae of bees determines whether the females shall be fertile queens or infertile workers. The marked differences in structure and instincts of the two classes of females are all conditioned by the food provided for the larvae. Each larva inherits the capacity to react in either way according to the stimulus received. Other examples from various animal and plant species might be cited to show how conditions in respect of light or moisture, as well as food or temperature, may determine the form which an organism will take.

In the algae and fungi, those closely related groups of organisms standing next above bacteria, life histories are extremely complex. Thousands of species of fungi are classed as *Fungi Im-*

perfecti, because the members are supposed to be stages in the life cycles of forms belonging to the Ascomycetes and Basidiomycetes. The final classification of the members of the *Fungi Imperfecti* must await a knowledge of their life histories.

Certain of the fungi having exceedingly complex life cycles have been carefully studied on account of their great economic importance. Among these are the Uredinae, or rust fungi, which attack grasses and other plants of economic importance. In certain of the rust fungi five different kinds of spores may develop in different stages of their life history. Over one hundred and fifty species of rust fungi are known to be heteroecious—that is, dependent on two distinct host plants for the completion of their life history. And, to add to the complexity, there are many species of rust fungi which are subdivided into races which cannot be distinguished morphologically, but which are physiologically distinct.

The well known species *Puccinia graminis* exhibits all the complexities just mentioned. It consists of several distinct races, all structurally alike, but showing peculiarities in the type of grasses on which they will grow. One will grow on rye or barley but not on oat; another will grow on wheat, barley, rye and oat, but not on other grasses, and the other races exhibit similar peculiarities. All the races of *Puccinia graminis* are heteroecious, with the aecidium stage growing on the barberry. In the spring the wheat rust makes its appearance as rust colored spots or streaks on the stalks and leaves of the immature plant. The colored spots are due to the presence of a layer of countless numbers of minute brown spores, the uredospores of the summer fruiting form. The uredospores are carried by the wind or insects to other wheat plants where they germinate, sending mycelium into the tissue, and developing the rust spots on the leaves and stalks. This phase of the life cycle, which requires about two weeks for its completion, is repeated throughout the summer, but toward the end of the summer the uredospores are replaced by the winter resting spores, called teleutospores, which are larger, thicker walled and darker in color. The teleutospores remain inactive on the straw until spring, when they germinate on manure

heaps or on moist ground and produce minute sporidia, which are conveyed by the wind to the alternate host, the barberry. In due time the fungus known as *Aecidium berberidis* appears on the barberry leaves in the form of small cluster-cups, each of which is filled with chains of orange-colored aecidiospores. Infection of the young wheat plants follows the scattering of the aecidiospores. The rust spots of uredospores develop, and the life cycle is completed.

The life history of the wheat rust is even more complex, however, than the outline just given displays. On the barberry, in addition to the fertile aecidiospores, there is produced also another kind of spore—the spermatia—which do not germinate. They are generally regarded as abortive male cells. Further, it is not only the gross structure which is involved in the various changes occurring in the life cycle of the Uredinae, but also the internal structure of the cells. The aecidiospores, the uredospore bearing mycelium, the uredospores and the young teleutospores all contain a pair of nuclei. Before the teleutospore reaches maturity the nuclei fuse, and the uninucleate condition continues through the sporidia, the aecidium-bearing mycelium and the spermatia.

Although the normal and complete development of the wheat rust is as outlined above, the cycle does not always follow the complete course. In Australia the barberry is an imported plant, and of rare occurrence, yet wheat rust is very abundant. The uredospore phase of the cycle, which is interrupted in temperate climates by the intervention of cold weather, continues indefinitely in the warmer Australian climate.

The case of life cycles in algae is of particular interest to bacteriologists on account of the close relationship between the Cyanophyceae, or blue-green algae and the Schizomycetes. Although there is a marked contrast in the method of nutrition of these two groups, their morphological affinity is acknowledged.

Of the thousands of species of algae which have been described, relatively few have been traced through their life cycle from spore to spore, owing to the aquatic habit of most of the species and the minute size of many of them. Consequently there is uncer-

tainty about life histories. Most algologists have accepted pleomorphism, but they differ in opinion concerning the extent of pleomorphism. Whether all algae are pleomorphic, and how many phases one species may pass through, are the questions on which the algologists disagree. Some claim that the simpler forms of algae pass through a number of distinct phases in their life history. Others admit that metamorphoses occur, but believe that the phases of a given organism are few in number. One investigator reports that *Protoderma viride* passes through a series of changes so varied that at different times it presents the characters of twelve different genera. Another investigator does not find so great a pleomorphism, but reports that *Raphidium* passes through phases represented by five different genera. A third investigator confirms the observation that *Raphidium* undergoes a metamorphosis by which it is converted into an organism with the characteristics of another genus, but was unable to observe further metamorphoses.

The striking contrast between the gradual changes which occur in the individual mammal, and the abrupt changes in morphology, accompanying changes in mode of life, which occur in lower forms corresponds with differences in exposure to environmental changes. In mammals, the life of the individual begins with an ovic cell, which develops during embryonic life in a carefully controlled environment with constant internal temperature, moisture and food conditions insuring a definite course of development. Throughout the life of the individual there is an interchangeability of body fluids among the various organs; and there is a dependence of form, rate and extent of growth and maturity upon internal secretions. The interdependence of one organ upon another secures a balance and continuity of development along prescribed lines with protection against disturbing influences of environment, which is in contradistinction to the conditions found in lower forms of life. In lower forms, as, for example, in insects, the mechanisms for maintaining constant conditions are less perfected than in mammals, but this defect is counterbalanced by a capacity for adjustment to variations in environment.

External influences operate most vigorously upon the one-

celled organisms, for in them the facility for an interchange of fluids is deficient or lacking. When one-celled organisms grow in masses, however, the individual cells influence and protect one another, as evidenced by the fairly common observation that bacteria sparsely seeded in a given medium may fail to grow, although they will grow in that particular medium if the seeding is more abundant. Whatever interchange of fluids there may be between isolated individuals of singled celled organisms is dependent on flux through the medium in which they are growing. Therefore the more complete the isolation of an individual, the more exposed it is to environmental factors.

In the examples cited of metamorphoses in two widely divergent forms of life, insects, on the one hand, and algae and fungi on the other, changes which occur in life cycles in response to environmental changes generally follow definite lines. With a given set of conditions the same form of the organism appears as appeared under those conditions in preceding years; and irregularities in environmental conditions result in irregularities in the life cycle.

Life cycles in bacteria should be expected, in accordance with the general biologic law that in the lower simple forms of life there is a capacity for irregular and sudden metamorphoses to adjust the organism to the vagaries of environment with special forms for special circumstances; and notable irregularities in the cycle should be expected in bacteria, because the influences of environment operate on exposed single cells.

If bacterial organisms follow life cycles of a complexity comparable to those of fungi, it will be an extremely difficult problem to demonstrate the changes. Bacteriologists will not quail, however, because they are confronted with difficulties. Every evidence of change in bacterial cultures should be examined with a mind ready to consider the possibilities of pleomorphism as well as to consider the possibilities of contamination. Let us therefore survey briefly the evidence of pleomorphosis and pleobiosis in bacterial organisms.

In his recent monograph on microbic dissociation, Hadley has ably considered the instability of bacterial species in such char-

acteristics as the kind of colony, antigenic power, agglutinability, and virulence. The changes which Hadley considered are, in general, changes which would implicate variety or species identification, according to present systems of classification. This discussion will be limited to the more profound changes of morphology and biologic behavior—changes of a magnitude comparable with the change from the uninucleate uredospore phase of the wheat rust, which can grow only upon grasses, to the binucleate aecidiospore phase which can grow only on the barberry. Changes of this order would move the organism from one genus or family to another, according to systems of bacterial classification now in use.

Metamorphosis is the term used by zoologists to describe the sudden changes in morphology which occur in life cycles. It is also used to describe sudden changes of morphology in the life cycles of the algae. The term has not been used by many bacteriologists, although it appears to be appropriate to describe bacterial changes which are comparable with the changes which occur in the animal kingdom and among algae.

As applied by zoologists to certain species, metamorphosis designates the changes through which a single individual organism passes, as for example, in butterflies. In other species, as in the trematodes, the cycle is spread over several generations, and the term metamorphosis is applied to the transformations which occur in the complicated development through the sporocyst, redia and cercaria stages, each stage representing a new generation, or series of generations descended from the adult trematode, and not three distinct genera, as was once supposed. The use of the term metamorphosis in bacteriology would be more comparable with the latter example.

Unfortunately the term mutation is used commonly by bacteriologists to describe the changes which occur in life cycles. The term mutation is generally applied by biologists to irreversible changes. Therefore it can not be correctly applied to the changes which occur in life cycles. It is probable that phylogenetic mutations giving rise to new taxonomic units occur in bacteria as in other forms of life, but until the changes that occur in

the ontogenetic life histories of bacterial organisms are known, it will be impossible to recognize an unusual change which could be correctly designated as a mutation. It is true that many investigators have reported that their mutated strains breed true indefinitely. In every such case, however, the unanswerable question arises whether the unknown conditions which might induce a reversion to the original type have been supplied.

Since the early days of the science, the theory of life cycles in bacteria has had its advocates. Billroth, Nägeli, Almquist, Adami, Hort and others recognized pleomorphism in bacteria. They were unable to assemble sufficient convincing data, however, to combat the prevailing monomorphic theory.

Löhnis reviewed the evidence of life cycles in bacteria which appeared in the literature up to 1918, and his monograph includes an extensive bibliography. In 1925 Enderlein continued the argument and presented further evidence in favor of life cycles.

New evidence of metamorphoses in bacteria appears from time to time and its value should be appraised. Let us examine a few of the most convincing observations which have been reported.

In 1909 Bredemann reported that it is possible to transform *Bacillus amylobacter*, an anaerobic spore-bearing bacillus, which vigorously ferments carbohydrates, into an aerobic, non-spore-bearing and non-fermenting coccus. He stated that he could induce this transformation in all his strains, but he was unable to revert the coccus form into the typical anaerobic bacillus. He was convinced of the purity of his cultures. In 1927 Cunningham and Jenkins confirmed Bredemann's observations. They studied six strains of *B. amylobacter* from as many different sources, and obtained coccus cultures from two of them by cultivation under incomplete anaerobiosis. Careful precautions were taken to ascertain the purity of the cultures. Fourteen strains of coccus cultures derived from *B. amylobacter* were studied. Two of them produced pigment, and agreed with the descriptions of *Micrococcus aurantiacus*. The remainder of the coccus cultures produced no pigment, and agreed with the descriptions of *Micrococcus candicans*.

By cultivating in sugar agar pure strains of a bifurcated, non-motile, non-spore-bearing anaerobic organism, Noguchi was able to induce its transformation into a motile, spore-bearing, aerobic bacillus; and by gradual training to the anaerobic life he was able to induce a reversion to the original phase of the organism. Howe and Hatch confirmed Noguchi's observations. They succeeded in producing a sporulating stage from the bifurcating form, and in transforming the spore-bearing to a non-spore-bearing stage.

While investigating the etiology of malignant granulomata, Negri observed remarkable pleomorphism in his cultures. In order to ascertain whether the transformations were due to contaminations, he obtained single cell cultures of the organism. With all precautions against contamination, he was still able to effect the transformations. He used the term metamorphosis to describe the changes which he observed. Various coccoid and diphtheroid forms could be induced, dependent on the nature of the medium on which the organism was grown. Very soon after Negri's report, Löhnis and Smith's *Life Cycles of the Bacteria* was published, in 1916. These workers studied the pleomorphism of Azotobacter and found that it undergoes a complicated life cycle. They then studied various other bacteria, and found that every species studied exhibited most of the forms found in Azotobacter. Löhnis and Smith acknowledged that their observations were essentially identical with those of Negri. Kellermann and Scales then took up the study of life cycles in bacteria. They studied twelve strains of *Bacillus coli (Escherichia coli)* from widely differing sources, and they observed in these cultures all the forms, excepting the spore-bearing, described by Löhnis and Smith.

In 1923 Löhnis and Smith reported further studies on 30 strains of Azotobacter. They found seven different cell forms, each with its own peculiar physiologic behavior. The various phases represented five different genera—*Micrococcus, Bacterium, Pseudomonas, Bacillus* and *Mycobacterium*.

In 1919 Mellon described a fuso-spirillary organism which grew as branching filaments, cocci or rods. There was also a filtrable phase. In a more recent publication Mellon emphasized the virulence of the filtrable or gonidial phase of the organism.

Mellon and Yost showed that *"Bacillus alkaligenes"*—known as a rod form—may grow in the human body as a Gram-positive diplococcus and that it can be cultivated as such on artificial media for years. It may also grow as a streptothrix, and there is another phase of mixed morphology.

Tunnicliff and Jackson cultivated from a tonsillar granule a pleomorphic organism which, in its most frequently observed and stable form, was a short bacillus. During the course of its life cycle it appeared also as large and small coccoid forms, and as straight and wavy filaments. The organism multiplied by fission, by budding, and by the production of gonidia.

In my study of the etiology of epidemic encephalitis I have made some observations which add to the evidence of metamorphoses in bacteria. I found that *Bacillus subtilis* and certain other common saprophytic bacteria are virulent when inoculated into the brains of rabbits. Some strains of *B. subtilis* are so virulent that they kill the rabbits within two or three hours if a small quantity of broth culture is inoculated into the brain. Other strains are less virulent, and, occasionally, one may be found which will produce symptoms from which the rabbit will recover within a few days. After a few weeks or a few months the recovered rabbit may develop symptoms of encephalitis and die after a brief illness. The brain of such a rabbit contains a virus which is indistinguishable from the well known so-called herpetic and encephalitic viruses. From the brains of rabbits which die following inoculation of this virus, and from human encephalitic material both *B. subtilis* and streptococci may be cultivated irregularly in chopped meat medium. In the first and second culture generations the bacterial organism is unstable, and metamorphosis may be observed. After a few transfers the form of the organism becomes stabilized, and will remain pure so long as it is maintained in culture media with occasional transfers. I have seen the transformation of rod to streptococcus so many times that I am forced to the conclusion that *B. subtilis* with its endospores is the resistant resting phase of an organism with a complex life cycle, certain phases of which are parasitic in mammals. The details of these experiments and observations will be published elsewhere.

This is not a proper occasion to continue the citation of instances of metamorphoses in bacteria. The extensive bibliographies in the monographs of Löhnis, Enderlein and Hadley are available to those who want to investigate the subject. The examples of metamorphosis already cited are sufficient to establish beyond a reasonable doubt that metamorphosis does occur in bacterial organisms. The questions now open to consideration are; whether all bacteria undergo metamorphoses; and through how many phases a single species may pass.

There are a number of striking coincidences of particular bacterial forms commonly found in certain diseases although the evidence indicates that they are not the primary cause of the disease with which they are associated. Thus, streptococci are associated with epidemic encephalitis, although some unknown virus is generally regarded as the cause of the disease; similarly, streptococci and "globoid bodies" are associated with poliomyelitis, a disease caused by a filtrable virus; and streptococci are also associated with measles, another disease in which an unknown filtrable virus is generally regarded as the cause; "Proteus X19" is associated with typhus, although apparently it does not possess pathogenic power; *Salmonella suipestifer* is associated with hog cholera, a disease caused by a filtrable virus; and the so-called *Bacillus bronchisepticus* is associated with the pneumonic type of dog distemper, although the virus of this disease, also, is filtrable. In the case of some of these conditions, the possibility that the associated bacteria may be a phase in the life cycle of the causal organism has already received consideration. The study of these diseases offers a field which may serve as the proving-ground for the pleomorphic theory.

Bacteriologists need not feel chagrinned if they have to admit that they cannot follow the life cycles of bacteria; and that forms they have considered as different genera are but stages in the life cycle of one species. There is solace in the thought that the difficulty in following a life history increases in inverse proportion to the size of the organism. Yet the zoologists once thought the larval stage of the lamprey was a separate genus, which they called *Ammocoetes*. The zoologists cannot arrive at a satisfactory

classification of the Hydromedusae because they do not know their complete life cycles; they do not in all cases know the polyp corresponding to a given medusa, nor the medusa that arises from a given polyp. The helminthologists cannot find the larval stage of certain cestodes parasitic in sheep; to this day the larval stage of the human tapeworm, *Taenia solium*, is called *Cysticercus cellulosae*. The mycologists readily admit their inability to discover the complete life cycle of a great number of species of fungi; and the algologists are in the same confused state of mind in regard to life cycles as are bacteriologists.

There is a story told of a New England philosopher who while driving through the country came upon a curious, low, rambling house in building. He went up to the house and inquired of a carpenter "Who is the architect of this building?" The carpenter replied, "We have had none yet, but we are going to send to Boston before long to get some one to come and put in the architecture."

The science of bacteriology is in some respects a rambling structure of incoordinated knowledge, and the time has come when it is at least as important to audit and correlate existing knowledge as it is to learn new isolated facts. A vast amount of information has been accumulated about this and that and the other form as they occur in particular habitats, but we are only beginning to appreciate the possibilities of the revelations which may await the study of life cycles in bacteria. The theory recently promulgated by Hadley that bacteriophage is a phase in the life cycle of bacteria adds a new conception involving practical applications.

A knowledge of life cycles would presumably influence industrial bacteriology the least of the several branches of the science, for the conditions maintained in industrial processes are more or less under control, and as a result the useful or harmful bacterial forms which are encountered are limited to those which can multiply under the given conditions. On the other hand, the significance of life cycles in bacterial organisms to soil science, phytopathology, medical bacteriology, immunology and epidemiology can only be imagined, for in all his relationships with bacteria in their natural habitats man is compelled to deal with all of their potentialities.

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A COLORIMETRIC METHOD FOR THE DETER-
MINATION OF BUTYRIC ACID[1]

R. J. ALLGEIER, W. H. PETERSON AND E. B. FRED

*From the Departments of Agricultural Chemistry and Agricultural Bacteriology,
University of Wisconsin, Madison*

The determination of the different kinds of volatile acid pro-
duced by bacteria is a problem that presents many difficulties.
Duclaux's method is the recognized procedure, but unfortu-
nately this method takes a great deal of time, requires very care-
fully standardized apparatus, and considerable skill and experi-
ence on the part of the operator. When large numbers of cultures
have to be examined for the purpose of sorting out those which
produce a particular acid, e.g. butyric, it is practically impossible
to use the Duclaux method. A more rapid method even though it
does not possess the accuracy of the Duclaux procedure is
decidedly helpful.

A qualitative colorimetric test for butyric acid in a mixture of
volatile acid was reported by Agulhon in 1913 and slightly modi-
fied by Dyer (1917). By employing more suitable conditions
for applying this test and by introducing a set of standard color
tubes we have made the method quantitative.

EXPERIMENTAL

The general principle of Agulhon's test is to convert butyric
acid into its copper salt and to extract this with ether. The
copper butyrate imparts a blue color to the ether layer. The
degree of color varies with the concentration of butyrate, the
acidity of the solution and the quantity of copper salt used.

With ether as solvent, precipitates frequently form and invalidate the test. We have found chloroform to be a more satisfactory solvent than ether, and by choosing the proper conditions, have overcome the formation of precipitates. After a large number of trials the following method has been developed.

Reagents

1. Copper chloride—hydrochloric acid reagent.
Dissolve 85.26 grams of $CuCl_2 \cdot 2H_2O$ in 1000 cc. of 1 N HCl.

TABLE 1

Composition of acetate-butyrate mixtures for preparation of standard color tubes

TUBE NUMBER	0.5 N SODIUM ACETATE	0.5 N SODIUM BUTYRATE	PERCENTAGE COMPOSITION	
			Acetic acid	Butyric acid
	cc.	*cc.*		
1	2.55	0.45	85	15
2	2.40	0.60	80	20
3	2.25	0.75	75	25
4	2.10	0.90	70	30
5	1.95	1.05	65	35
6	1.80	1.20	60	40
7	1.65	1.35	55	45
8	1.50	1.50	50	50
9	1.35	1.65	45	55
10	1.20	1.80	40	60
11	1.05	1.95	35	65
12	0.90	2.10	30	70
13	0.60	2.40	20	80
14	0.30	2.70	10	90

2. Chloroform—C. P.
3. Standard color tubes.

Make up half normal solutions of sodium acetate and sodium butyrate as follows:

Dilute 3 grams of glacial acetic acid with 20 cc. of water and neutralize to phenolphthalein with 1 N NaOH. Dilute the resulting sodium acetate solution to twice the volume of the 1 N NaOH used. This gives a solution of exactly 0.5 N strength.

Redistill butyric acid (Eastman Kodak Company) and collect

the fraction boiling between 161 to 162°C. Dilute 4.4 grams of this distillate with water and treat in the same manner as above to give a 0.5 N strength solution of sodium butyrate.

Mixtures of these two solutions are made as indicated in table 1. The combinations are made in a separatory funnel and to each combination 0.4 cc. of $CuCl_2$-HCl reagent are added and the mixture shaken. Five cubic centimeters of chloroform are then added and shaking continued until a maximum color develops in the chloroform layer (twenty to thirty seconds). After the chloroform layer has settled it is drawn off into test tubes of uniform diameter and closed with a well-fitting cork of good quality. The cork is cut off level with the top of the tube and covered with a 10 per cent solution of gelatin. The level of the liquid in the tube should be marked with a file so that if evaporation does take place it may be detected.

Preparation of sample for analysis

If the volatile acids are contained in cultures the following procedure is used for their removal.

To 50 cc. of the culture (or a quantity that will give at least 3.0 cc. of 0.5 N volatile acid) add 20 grams of $NaH_2PO_4 \cdot H_2O$, 15 cc. of 85 per cent H_3PO_4 and distill off the volatile acids. With a blue pencil draw a line on the distilling flask at 50 cc. volume and keep within 10 to 15 cc. of this volume during the distillation by adding water from a dropping funnel at about the same rate as the liquid distills.

Collect 200 cc. of distillate which will contain practically all of the volatile acid (Fred et al., 1919). Titrate the distillate with 0.5 N or other standard NaOH to the end point of phenolphthalein and evaporate to such a volume as will be slightly stronger than a 0.5 N solution. If the solution shows any traces of pink color, neutralize with HCl and dilute to an exactly 0.5 N solution.

To exactly 3 cc. of the neutral solution add 0.4 cc. of $CuCl_2$-HCl reagent and shake. Then add 5 cc. of chloroform and shake until a maximum coloration develops in the chloroform layer. After settling, the chloroform layer is drawn off into a test tube of the same diameter as the standard tubes and matched against the standards.

Source of light for comparing tubes

Comparison of the unknown with the standard tubes is best accomplished by the use of electric light. The most suitable source of light was found to be a white frosted bulb placed in a metal container. A small opening, 1 by 1 inch, was cut in this container and the opening covered with a piece of onion skin typewriter paper. The unknown and standard are held close to this opening and compared. The comparison is repeated with various standards until the best possible match is obtained. If the unknown falls between two of the standards, the value of the unknown is interpolated.

If the unknown solution contains more than 70 per cent of the total acid in the form of butyric, it is better to use 0.25 N solution of both standards and unknown, and to reduce the quantity of $CuCl_2$-HCl reagent to 0.2 cc. Owing to the intensity of color produced it is difficult to match the higher concentrations of butyric acid. If the unknown contains less than 20 per cent of the acid in the form of butyric, the method can be made more sensitive by using 1 N solutions of both standards and unknown and by increasing the quantity of $CuCl_2$-HCl reagent to 0.8 cc. In any case the smallest quantity of butyric acid that can be detected is about 20 mgm.

Factors influencing color

Concentration of $CuCl_2$ and HCl. The color test is presumably based upon the following reaction:

$$2 \, C_3H_7CO_2Na + CuCl_2 \rightleftharpoons (C_3H_7CO_2)_2 \, Cu + 2 \, NaCl$$
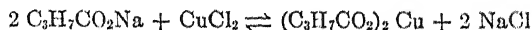
$(C_3H_7CO_2)_2Cu$ is soluble in certain organic solvents and imparts a blue color to the solvent. Secondary reactions seem to occur due perhaps to the hydrolysis of the $(C_3H_7CO_2)_2Cu$ with the formation of basic insoluble compounds. To prevent the formation of these precipitates and to insure the maximum of color a certain amount of free acid is necessary.

In the course of developing the method which has just been described a number of tests were conducted to ascertain the most

suitable conditions for the procedure.   Table 2 shows the change in color and effect on precipitate formation by varying the quantities of $CuCl_2$ and HCl.   All the tubes contained the same quantity of volatile acid, 3 cc. of 0.5 N.   In the first series this

TABLE 2

Effect of acidity and concentration of copper chloride on color intensity in chloroform layer

TUBE NUMBER*	1 N HCl	2 N CuCl₂	APPARENT CONTENT OF BUTYRIC ACID		FORMATION OF PRECIPITATE
			Series 1	Series 2	
	cc.	*cc.*	*per cent*	*per cent*	
1	0.30	0.15	40	80	None in 3 months
2	0.45	0.15	40	80	None in 3 months
3	0.60	0.15	34	73	None in 3 months
4	0.40	0.10	31	60	None in 3 months
5	0.40	0.20	40	87	None in 3 months
6	0.40	0.30	47	100	After 27 days
7	0.40	0.50	55	100+	After 12 hours
8	0.60	0.20	38	77	None in 3 months
9	0.60	0.30	43	100	None in 3 months
10	0.60	0.50	45	100+	After 48 hours
11	0.80	0.20	33	73	None in 3 months
12	0.80	0.40	33	73	None in 3 months
13	0.80	0.60	33	73	After 24 hours
14	0.80	0.50	33	73	None in 3 months
15	0.80	0.50	33	73−	After 3 days
16	0.80	0.80	33	73	After 12 hours

* Both standards and tubes under test contained 3 cc. of 0.5 N acid.   Of this acid 40 per cent was butyric in Series 1 and 80 per cent in Series 2.   The standards contained 0.4 cc. of 1 N HCl and 0.15 cc. of 2 N CuCl₂; the tubes under test contained the quantities indicated in columns 2 and 3.

consisted of 60 per cent acetic and 40 per cent butyric acid; in the second series the proportion was 20 per cent acetic and 80 per cent butyric acid.   After adding the HCl and $CuCl_2$, water was added to make a constant volume of 4 cc. and the solution was then shaken with 3 cc. of chloroform.   Comparison of the color pro-

duced was made with standards similarly prepared but containing 0.4 cc. 1 N HCl and 0.15 cc. 2 N CuCl$_2$.

The data in table 2 show that with a fixed quantity of CuCl$_2$ the color in general decreased as the HCl increased. If nos. 1 and 3, and nos. 5 and 11 are compared, it is seen that doubling the quantity of HCl decreased the apparent percentage of butyric acid from 6 to 14 per cent.

If the HCl is kept low and constant, the color is increased as the CuCl$_2$ is increased—see nos. 4 to 10 inclusive. The effect is more pronounced with the higher percentages of butyric acid than with the lower. A limit is soon reached, however, as the formation of a precipitate sets in, and makes comparison impossible. There is no advantage in increasing the quantity of CuCl$_2$ above a certain point, since the deep blue color developed with the higher percentages of butyric acid becomes too intense for comparison.

Many other combinations of HCl and CuCl$_2$ were tried and on the basis of these tests, the combination in no. 5 was adopted as having the most desirable features over the whole range of acetic and butyric acid mixtures. This is the combination used in the standard color tubes and unknown solutions.

In the preliminary tests separate solutions of 2 N CuCl$_2$ and 1 N HCl were used. This did not prove satisfactory as the aqueous solution of 2 N CuCl$_2$ developed a yellow precipitate, presumably an oxide of copper, upon standing a few weeks. This reduced the concentration of the solution rendering it useless for the test. To avoid this effect, the CuCl$_2$ and HCl were combined and used as one reagent. No precipitate resulted with this combination and a saving of time in making the test was effected.

Effect of other acids. The presence of propionic acid produces high results, inasmuch as copper propionate is partially soluble in chloroform and produces a blue color. Several solvents were used in an attempt to eliminate this factor but to no avail. Also the Haberland (1899) method for the separation of propionic acid was tried but failed to give satisfactory results. Fortunately propionic acid is usually not formed by the bacteria that produce butyric acid. In pure culture work interference by propionic acid is, therefore, not a serious matter, but in mixed cultures the method becomes inapplicable.

Small amounts of formic acid, up to 25 per cent of the total, do not affect the determination, but larger quantities give low results. In cultures which produce butyric acid it is probable that formic acid to the extent of 25 per cent of the total would never be formed.

Use of other metallic salts and solvents. Cupric sulphate, cupric chloride, ferric chloride and cadmium chloride were tried in an attempt to find a suitable salt which would react with sodium butyrate giving a characteristic color to the solvent layer. Of these cupric chloride was found to produce the best results.

The following solvents were tested to find a suitable medium for color comparisons: carbon tetrachloride, carbon disulphide, petroleum ether, ethyl ether, chloroform, xylene, benzene, benzaldehyde, octyl alcohol, amyl alcohol, amyl acetate and ethyl acetate. A blue color developed in all cases except with carbon tetrachloride and carbon disulphide. Ether and chloroform gave the maximum and about the same intensity of color. Ether was found to be objectionable because of the tendency to form emulsions and the slowness with which the two layers separated. Moreover, precipitates and a fading of color resulted even when the ether layer was separated from the mixture. The chloroform layer, on the other hand, separated almost immediately and showed less tendency to form precipitates. Chloroform possesses another advantage in that it does not evaporate as readily as ether and thus permits the preparation of permanent standards.

Comparison with the Duclaux method

The colorimetric method was checked a number of times with mixtures of pure acetic and butyric acids and found to give accurate results. It was then tried out on cultures of butyric acid-forming bacteria. Acids from two types of such microörganisms were tested. One group is made up of the acetone-butyl alcohol microörganism, represented by cultures 70 and 105 and the other group consists of the non-butyl alcohol-producing bacteria and includes a number of widely differing bacteria which are designated in the table by nos. 2, 9, 21, 22,

24, 25, 31, 32, 36. In all seventeen cultures were used and the results by the two methods are given in table 3. The difference between the two methods ranged from +6.0 per cent to −6.5 per cent. This degree of accuracy is probably sufficient for most

TABLE 3

Comparison of the colorimetric and Duclaux methods for the determination of butyric acid in bacterial cultures

CULTURE NUMBER	BUTYRIC ACID BY		DIFFERENCE
	Colorimetric method	Duclaux method	
True butyric acid bacteria			
	per cent	*per cent*	*per cent*
2	27.0	28.4	−1.4
9	49.0	54.1	−5.1
21	66.0	65.4	+0.6
22	66.0	70.3	−4.3
22	77.0	73.1	+3.9
24	73.0	76.8	−3.8
24	73.0	74.0	−1.0
25	80.0	79.5	+0.5
25	60.0	66.5	−6.5
31	57.0	59.8	−2.8
32	73.0	71.2	+1.8
36	60.0	65.2	−5.2
36	58.0	59.0	−1.0
36	80.0	74.0	+6.0
Acetone butyl-alcohol bacteria			
70	47.0	43.3	+3.7
105	56.0	54.5	+1.5
105	48.0	44.6	+3.4

fermentation work and in the sorting out of large numbers of butyric acid-producing bacteria should save a great deal of time.

SUMMARY

A colorimetric method for the estimation of butyric acid in the presence of acetic and formic acids is described. The solution to be tested is made to a definite strength and is then treated

with a $CuCl_2$-HCl reagent and the mixture shaken with chloroform. Known mixtures of butyric and acetic acids are treated in exactly the same manner and are used as standards for comparison with the unknown. By carefully standardizing the procedure good checks were obtained with the Duclaux method on 17 cultures producing butyric acid.

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BACTERIAL ANTAGONISM WITH SPECIAL REFERENCE TO THE EFFECT OF PSEUDOMONAS FLUORESCENS ON SPORE FORMING BACTERIA OF SOILS

I. M. LEWIS

Departments of Botany and Bacteriology, the University of Texas

Bacterial antagonism has been extensively studied in the past by many investigators. The voluminous literature of the subject is fully reviewed in Kolle and Wassermann's *Handbuch* and requires but brief consideration here.

Pseudomonas fluorescens, Ps. putida and *Ps. pyocyanea* are frequently mentioned in the literature as organisms which exert an inhibitory or bactericidal action on other species of bacteria. There are conflicting reports, however, as to the organisms inhibited and concerning the nature and cause of antagonism. As early as 1887, Garre studied the effect of *Ps. putida* on several species of bacteria. He found that *Staph. aureus, B. typhosus,* and *B. mucosus-capsulatus* were inhibited while *B. anthracis* and the vibrio of Finkler and Prior grew normally. These results were not in complete accord with those of Lewek (1889) who found that in association with *Ps. putida, B. anthracis* and *Staph. aureus* were both killed, *Staph. albus* grew feebly, *V. cholera* was retarded while *B. typhosus* and *B. coli* grew vigorously. *Ps. fluorescens* had no effect on *B. typhosus, Staph. aureus* or *V. cholera* but was strongly antagonistic for *B. anthracis.* Olitsky (1891) determined that *B. typhosus, B. anthracis, V. cholera, B. prodigiosus* and *Staph. aureus* are markedly inhibited by *Ps. fluorescens* These results are at variance with those of Garre as to the effect on *Staph. aureus* and *B. typhosus.*

The survival of *B. typhosus* in sewage was studied by Laws and Andrewes (1891) who found that the period was shortened in the presence of *Ps. fluorescens.* Horrocks (1901) was unable to

recover *B. typhosus* after it had been incubated for seven days with *Ps. fluorescens* in sterilized sewage. Frost (1904) found the metabolic products of both *Ps. fluorescens* and *Ps. putida* to be not only inhibitory but also bactericidal for *B. typhosus*.

Other investigators, not concerned primarily with the question of antagonism, have cultivated *Ps. fluorescens* in mixed association with other species. Luxwolda (1911) studied the growth of *Ps. fluorescens* and *Strep. lacticus* separately and in mixed association in milk at different temperatures. At temperatures of 20, 15, 13 and 10°C., both species appeared to be benefited by the association.

Conn and Bright (1919) inoculated sterilized manured soil with a mixture of *B. cereus* and *Ps. fluorescens* cells and made subsequent quantitative analyses for a period of fifteen days to determine the relative rate of multiplication. They failed to obtain colonies of *B. cereus* in the dilutions plated while *Ps. fluorescens* had multiplied enormously. The same result was obtained when the ratio of cells in the inoculum was greatly in favor of *B. cereus*. Similar results were obtained in unsterilized manured soil. *B cereus* failed to increase appreciably under the conditions of these experiments.

Waksman and Lomanitz (1924) cultured *B. cereus* and *Ps. fluorescens* separately and in mixed association in flasks of casein solution. They found that *B. cereus* was very active in this medium while *Ps. fluorescens* failed to attack the substance vigorously when growing alone. In the flask inoculated with the two species, it was found that *B. cereus* decomposed the casein, producing amino acids which were further decomposed by *Ps. fluorescens*, as soon as formed, with the production of ammonia. The exact quantitative bacterial analysis of the mixed culture is not given in the report of these authors. They state, however, "the number of *Ps. fluorescens* cells greatly exceeded those of *B. cereus;* the culture looked and smelled like a typical *Ps. fluorescens* culture."

The present investigation was undertaken for the purpose of determining more fully the effect of *Ps. fluorescens* on some species of spore-forming soil bacteria and what influence if any is exerted

on their development in soil by the bacterio-toxic substance. Additional species were included in the experiments in order to determine relative susceptibility.

EXPERIMENTAL METHODS

Various methods have been used for the study of bacterial antagonism. The method of Garre (l.c.) has been employed extensively by others, either in its original form or with slight modifications. Agar or gelatin is poured into Petri dishes, allowed to harden and inoculated on the surface with the two species placed alternately either parallel or radiating from the center.

This method has some value but it is generally less satisfactory than other methods. Very striking results are obtained when a spreading form such as *B. mycoides* is grown in association with *Ps. fluorescens*. As the filamentous rhizoid-like growth approaches the inhibitory zone it halts abruptly and is unable to make a further advance. The growth of *B. mycoides* on the side adjacent to the inhibitor contrasts sharply with that on the opposite side. This method affords the advantage of permitting either simultaneous inoculations or a period of time for the growth of the inhibitor.

A more useful plate method of cultivating two species in mixed association is the seeded plate with surface inoculation. The agar, seeded with one of the pair, is poured into a Petri dish and inoculated on the surface with the other.

The results are striking when such a strongly inhibiting organism as *Ps. fluorescens* is grown on the surface of agar seeded with an inhibited species which has a slow normal rate of development. The extent of the inhibited zone depends on several factors, of which the composition and consistency of the agar, its depth in the dish, amount of inoculum, susceptibility of the inhibited species and the normal growth rate of the two species appear to be the more important.

When agar is seeded, not too heavily, with spores from an old culture of *B. mycoides* and inoculated on the surface with *Ps. fluorescens* the zone of inhibition reaches a width of 2 or 3 cm.

(fig. 2). In plates seeded with young cultures containing actively growing cells the results are much less marked. In such slow growing species as *Sarcina lutea* and *Lactobacillus flavus* the zone in which colonies fail to develop extends almost the full width of the dish. Time is required for the accumulation of metabolic
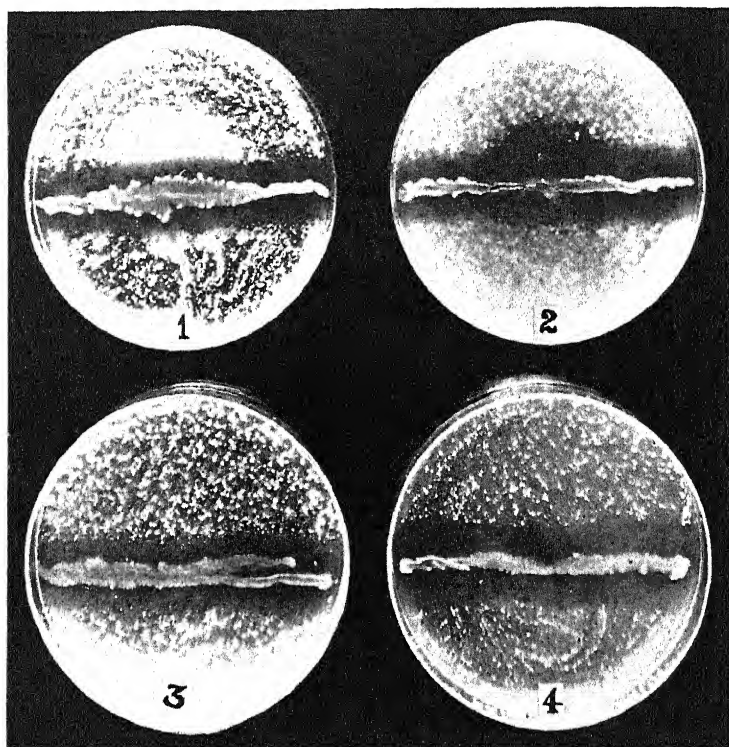

Fig. 1. B subtilis   Fig. 3. B vulgatus
Fig. 2. B. mycoides   Fig. 4. B. cereus

products in sufficient concentration to check development of the colonies. With such species as *B. coli, Serratia marcescens* and others the colonies are already developed before this point is reached.

The seeded plate method is useful for preliminary reconnaissance, but its application to the study of bacterial antagonism is

limited and the results, when negative, are very often misleading. For the more precise study of antagonism other methods are preferable. The collodion sack method of Frost gives reliable results but it is difficult and time consuming when many tests are to be made.

In the present investigation, the seeded plate method with surface inoculation was used for preliminary experiments. In order to determine more definitely the degree of inhibition, old cultures of *Ps. fluorescens* were sterilized and added to nutrient agar in various proportions. The agar base was made of proper strength to contain 0.5 per cent peptone, 0.3 per cent beef extract, and 2.0 per cent agar after the addition of the sterilized old culture. The final reaction was adjusted to pH 7.2. The finished agar was poured into Petri dishes and inoculated on the surface with the organisms to be tested.

A suitable culture medium for the production of toxin was determined by experiment. It was considered desirable to use a synthetic medium if possible. Media prepared according to the formulae of Uschinsky (1893), Jordan (1899), Braun and Cahn-Bronner (1921) and Fraenkel (1894), as well as a variety of nonsynthetic media were tested. The latter included casein and blood digest; peptone; peptone plus beef extract; peptone plus gelatin, and peptone, beef extract plus glucose. The toxic substance was developed in all media but was found to be more abundant in Uschinsky's medium. Peptone beef extract agar was used for the seeded plate method and either Uschinsky solution or agar for all other experiments.

The strain of *Ps. fluorescens* was isolated from water. It gave all the typical reactions for the species and produced abundant pigment in all suitable media. The test organisms were principally stock cultures carried in this laboratory but some were freshly isolated from original sources.

EFFECT OF PSEUDOMONAS METABOLITES ON OTHER SPECIES OF BACTERIA AND FUNGI

Preliminary experiments, in which the seeded plate method was employed, showed that some species are strongly inhibited while

TABLE 1

NAME OF ORGANISM	PER CENT OF AGED MEDIUM IN THE AGAR (PLUS SIGN DENOTES COMPLETE INHIBITION)									
	0.5	1.0	2.5	5.0	10	15	20	30	40	50
B. cereus	−	−	+							
B. mycoides	−	−	+							
B. anthracis	−	+								
B. sp. Number 27	−	−	+							
B. Sp. Number 30	−	−	+							
B. vulgatus	−	−	+							
B. subtilis	−	−	+							
B. megatherium	−	+								
Rhodococcus cinnebareus	−	+								
Rhodococcus roseus	−	−	+							
Micrococcus flavus	−	−	−	+						
Neisseria catarrhalis	−	−	−	+						
Ps. aeruginosa	−	−	−	−	−	−	−	−	−	−
Ps. fluorescens	−	−	−	−	−	−	−	−	−	−
Sarcina lutea	−	−	−	+						
Sarcina aurantiaca	−	−	+							
Serratia marcescens	−	−	−	−	−	+				
Staph. albus	−	−	+							
Staph. aureus	−	−	−	+						
Staph. citreus	−	−	+							
Encapsulatus pneumoniae	−	−	−	+						
Vibrio comma	−	+								
Chromobacterium violaceum	−	+								
Eberthella typhi	−	−	+							
Eberthella paradysenteriae Hiss	−	−	+							
Eberthella paradysenteriae Flexner	−	−	+							
Salmonella enteritidis	−	−	−	+						
Salmonella morgani	−	−	−	+						
Salmonella paratyphi	−	−	−	+						
Salmonella suispestifer	−	−	−	+						
Salmonella schotmulleri	−	−	−	+						
Salmonella pullorum	−	−	−	+						
Salmonella gallinarum	−	−	−	+						
Escherichia coli	−	−	−	−	−	−	+			
Aerobacter aerogenes	−	−	−	−	−	−	+			
Phytomonas bowlesii	−	−	+							
Saccharomyces marianus	−	−	−	−	−	−	−	−	−	
Saccharomyces intermedius	−	−	−	−	−	−	+			
Saccharomyces ellipsoideus	−	−	−	−	−	−	−	+		
Saccharomyces pastorianus	−	−	−	−	−	−	−	−	+	
Schizosaccharomyces mellacei	−	−	−	−	−	−	+			
Zygosaccharomyces priorianus	−	−	−	−	−	−	−	+		
Torula sphaerica	−	−	−	−	−	−	−	−	−	
Aspergillus niger	−	−	−	−	−	−	−	−	−	
Aspergillus ochraceus	−	−	−	−	−	−	−	−	−	
Rhizopus nigricans	−	−	−	−	−	−	−	−	−	
Alternaria sp	−	−	−	−	−	−	−	−	−	
Fusarium sp	−	−	−	−	−	−	−	−	−	
Penicillium sp	−	−	−	−	−	−	−	−	−	

others are not influenced by the surface growth. The species which showed the most marked inhibition were the spore forming organisms from soils and various micrococci. *B. coli* and *Serratia marcescens* were not inhibited. Results obtained by this method are shown in the accompanying illustration. Plain extract agar was seeded with the various organisms and inoculated on the surface with *Pseudomonas fluorescens*. The cultures were seventy-two hours old when photographed (figs. 1–4).

It was deemed desirable to determine not only whether or not a given species is inhibited by the metabolic products but also the minimum amount of such products necessary to cause total inhibition of growth. Accordingly a series of agars was prepared each containing a different per cent of aged medium. The culture of *Ps. fluorescens*, grown on the surface of sloped Uschinsky agar for a period of ten days, was sterilized at 15 pounds pressure and added to the nutrient agar base. The effect on a variety of test species is shown in the accompanying table 1.

It is seen that the antagonistic action is not specific but that some species are more resistant than others. This is especially true of *Ps. fluorescens* and *Ps. pyocyanea*. The most sensitive species were found to be *B. anthracis*, *B. megatherium*, *Vibro comma*, *Chromobacterium violaceum* and *Rhodococcus*. Other species of *Bacillus*, *Eberthella*, *Sarcina*, *Neisseria* and *Phytomonas* are only slightly more resistant. The species of *Salmonella* are less sensitive while *Escherichia coli*, *Aerobacter aerogenes* and *Serratia marcescens* are relatively strongly resistant. All of the fungi grew normally while some of the yeasts were inhibited by amounts of 20 to 50 per cent.

Further evidence on the sensitiveness of bacteria and fungi was obtained by plating soil dilutions in the various grades of agar, prepared as above plus 0.5 per cent glucose. On plates prepared from dilutions of 1:1000 and 1:10,000, colonies of bacteria, except green fluorescent species, failed to develop on the grades above 20 per cent. The number and variety of mold colonies was practically the same as on the acid synthetic agar of Waksman's formula. Actinomycetes appeared to be more sensitive than fungi since very few colonies were observed. It

appears probable that all bacteria except the green fluorescent forms are inhibited while fungi do not appear to be affected. It is obvious that inhibition is not due to the reaction of the medium or to lack of food but to a thermostable toxic substance.

It seems probable that differences in resistance to the toxin depend, at least in part, on varying degrees of permeability. It is known that the cell wall of *B. cereus* is, in general, much more permeable than that of *B. coli*. Shaughnessy and Winslow (1927) have suggested that this phenomenon is probably related to the unequal periods of survival of these organisms in aqueous suspensions. The precise chemical nature of the bacterio-toxic substance produced by *Ps. fluorescens* is not known. It is water soluble and dialyzable through collodion and cell membranes and inhibits *B. cereus* and related forms in less amounts than are required for *B. coli*.

CONDITIONS WHICH INFLUENCE PRODUCTION OF THE TOXIN

It is well known that the development of true bacterial toxins depends on the composition of the culture medium and growth conditions. Previous investigations by Gunst (1923) and Lode (1903) have shown that the production of inhibitory and bactericidal substances by other species is influenced by the amount of available oxygen.

In order to test this effect, cultures of *Ps. fluorescens* were grown in Uschinsky's solution so arranged as to afford different exposures of air. The maximum exposure was obtained by placing 50 cc. of the medium in a 500 cc. Erlenmeyer flask. A similar flask was filled well up in the neck for comparison. The surface of the medium in each flask became covered with a dense zoogleal mass. The green fluorescent pigment developed more promptly in the shallow layer but at the end of the incubation period it had faded to a deep reddish green while there was little or no change of color in the deep flask. On agar containing 5 per cent of sterilized medium from the shallow layer, none of the test species grew while all of the same species developed on agar prepared by dissolving 0.5 peptone, 0.3 beef extract and 2.0 per cent of agar in the undiluted staled medium from the deep

layer. Proper aeration appears therefore to be a most important factor in the development of the toxic substance.

EFFECT OF VARIOUS AGENTS ON STABILITY

The bacterio-toxic substance produced by *Ps. fluorescens* is thermostable. Old agar cultures were sterilized at 100° and 120°C. for fifteen minutes and at 120°C. for thirty minutes. On nutrient agars containing 5.0 per cent of each, the sensitive species failed to develop.

The effect of desiccation was tested by saturating filter paper with Uschinsky's solution in which the organisms had grown for fourteen days. As soon as the paper became thoroughly dried the minimum amount necessary to cause complete inhibition was determined by adding various sized pieces to nutrient agar which was then sterilized and inoculated with a variety of species. A strip two by three inches added to 25 cc. of agar was sufficient to inhibit all of the spore formers and the sensitive cocci. The saturated paper was stored at temperatures of 58°, 37°, 30° and 10°C. and tested at intervals of ten days for a period of forty days. At the end of this period no deterioration had occurred.

The toxin is quite stable with age. Cultures grown on sloped Uschinsky agar were kept at room temperature for a period of thirty days and in the ice box for an additional sixty days. Evaporation was prevented by sealing with paraffin after the cultures were about three weeks old. On agars containing various amounts of the aged culture it was found that 3.5 per cent completely inhibited growth of all the spore formers and micrococci. The liquid medium loses its toxin more rapidly than agar. A culture kept forty-five days at room temperature had become completely non-toxic.

The toxin is not removed by filtration through Berkfeld or Seitz filters. Inhibition of the same species occurred when filtered or heated broth was added to nutrient agar in equal amounts.

BACTERICIDAL EFFECTS

Sterilized cultures grown in Uschinsky's solution failed to exert bactericidal effects against any of the species tested. In order

to test the effect of the unheated toxin, bits of agar removed from the inhibited zone of seeded plates were planted in nutrient broth. None of the spore forming species were killed while *Staph. albus Staph. aureus, Staph. citreus, B. typhosus B. paratyphosus A, B. paratyphosus B, Rhodococcus cinnibar* and *Sarcina lutea* failed to develop after a period of three to five days.

PRODUCTION OF TOXIN IN MANURED SOIL

The investigations of Conn (1917) and Conn and Bright (l. c.) have shown conclusively that *B. cereus* is not able to compete successfully with *Ps. fluorescens* in manured soils. To what extent this failure is due to the inhibiting metabolic products is open to question. It seems reasonable to suppose that under optimum conditions for the growth of *Ps. fluorescens* such inhibition might become pronounced. The very small amount of aged medium necessary to cause total inhibition of growth indicates that this is a probable explanation.

The hypothesis that soils frequently contain toxic substances which inhibit the growth of the higher plants and soil microorganisms appears to be quite generally accepted. It is known that such toxic effects may be removed by various methods of treatment such as oxidation, partial sterilization by heat, treatment with volatile antiseptics, the addition of certain substances and the adsorptive action of carbon black.

The precise mode of origin of soil toxins has not been conclusively demonstrated although there seems to be a more or less uniform opinion that such substances are not excreted as such by the roots of higher plants. It seems quite probable that soil microörganisms play an important part in their formation. Various workers have made comparative studies on the toxicity of soil extracts and bacteriotoxic substances produced by organisms when grown on artificial culture media. Frost (1904) was unable to obtain bactericidal effects against *B. typhosus* by any of the soil extracts tested but when pure cultures of various species isolated from the same soils were grown in broth, the effect was pronounced. He concluded that bactericidal sub-

stances do not exist preformed in soils, but are produced by various soil bacteria in artificial cultures.

Greig-Smith (1914) found that soils at times contain substances which are not favorable for the growth of *B. prodigiosus*. Extracts from the surface layer proved to be thermolabile while the sub-soil extracts were thermostable. His experiments showed that the soils with which he worked contained a variable bacteriotoxic content. Following rains which washed the substances down into the sub-soil the surface layers become non-toxic. The toxins were formed again as the soil dried out and persisted so long as suitable soil moisture was present. In very dry soil the toxin decayed. Experiments in the laboratory showed that a soil originally toxic became nontoxic when washed with water and upon incubation again became toxic. Such results would appear to point strongly, if not conclusively, to the origin of the bacterio-toxic substances through the activity of soil microorganisms.

In subsequent investigations Greig-Smith (1918) compared the effect of substances produced by microörganisms in cultures grown under various conditions with the soil extracts previously reported. Such inhibitions of *B. prodigiosus* as became manifest could be accounted for by changes in the hydrogen ion concentration of the medium and differed therefore from the toxic effects noted in soil extracts.

Hutchinson and Thaysen (1918) examined several samples of soil for the purpose of determining whether or not the beneficial effects of partial sterilization are due to the destruction of bacterio-toxins. Some of the soil extracts were found to be more suitable for the growth of *B. prodigiosus* than others. The unfavorable extracts were improved by the addition of peptone or antiseptics but not by heating. They found also that continued cultivation of *B. prodigiosus* in a solution produces an unfavorable medium which is not improved by heating. They regard this substance as different from the soil toxins reported by others.

In order to determine whether the toxin is produced in manured soils, cultures of *Ps. fluorescens* were prepared in soil to which 5.0 per cent of cotton seed meal or fresh horse manure was added.

The manured soil was placed, in 100 gram amounts, in Erlenmeyer flasks of 500 cc. capacity, sterilized by heating two hours at 15 pounds pressure, inoculated with *Ps. fluorescens* and incubated at room temperature for a period of fourteen days. Water extracts were prepared by adding 100 cc. of distilled water, shaking thoroughly at ten minute intervals for about two hours and then filtering through paper. Agar was prepared from the filtrates by the addition of 0.5 per cent peptone, 0.3 per cent beef extract and 2 per cent agar. The finished medium was sterilized at 15 pounds pressure and inoculated with various test species.

The manured soils failed to yield toxic substances in sufficient amount to inhibit the most sensitive of the test organisms. This seems to indicate that the bacterio-toxic substance is not produced under the conditions established or that it is adsorbed by substances in the soil and is not readily extracted by water.

It is known that soil toxins as well as the bacterio-toxins produced in cultures are adsorbed by charcoal and other substances. Gundel (1927) found that a thermolabile, non-filterable inhibiting substance produced by *B. coli* and antagonistic to *B. anthracis* is adsorbed by charcoal and several other compounds. Pratt (1924) found that the inhibitory effect of broth aged by *Fusarium* is removed by charcoal.

The power of charcoal and of field soil to adsorb the toxin from old cultures of *Ps. fluorescens* grown in Uschinsky solution has been tested in this investigation. The substances were placed in flasks and saturated with the solution. After standing twelve hours a quantity of distilled water equal to that of the original aged culture medium was added. The flasks were shaken thoroughly and the contents filtered through paper. The filtrates had lost most of the pigment which was originally present. This was especially pronounced in the case of the charcoal. Agars were prepared by dissolving the standard amounts of peptone, beef extract and agar at 15 pounds pressure. All test organisms, including the sensitive spore formers and cocci, developed normally thus showing that the toxic substance is not readily extracted from soils in which it is known to be present in abundance.

It is possible to extract some of the adsorbed toxin by means of alcohol. The charcoal was dried at 60°C. and extracted twelve hours. The filtrate obtained by filtration through paper was evaporated to dryness at 60°C. The dry residue was then re-dissolved in nutrient broth. On agar prepared from the broth the more sensitive species such as *B. cereus, B. mycoides, B. anthracis* and *Sarcina lutea* failed to grow. *B. coli* and *Serratia marcescens* were not inhibited.

With these data at hand, a second attempt was made to extract the toxin from sterilized manured soils which had been inoculated with *Ps. fluorescens.* The extracts were prepared in the manner described above. The alcoholic filtrate obtained from 100 grams of soil was evaporated to dryness at 60°C. and redissolved in 25 cc. of distilled water. On nutrient agar prepared from this water and standard amounts of nutrients, the more sensitive species failed to grow, while resistant forms such as *B. coli* and *Serratia marcescens* were not inhibited. Control flasks containing the same soil, sterilized but not inoculated, failed to yield toxin. Soil extracts prepared in the same manner from ten samples of field, garden, pasture and woodland soils were not toxic. None of these soils had been freshly manured and none contained large numbers of *Ps. fluorescens* cells.

Whether or not the toxin produced by *Ps. fluorescens* in soils is adsorbed to such a degree as to neutralize its inhibitory effect on bacteria in the soil might be questioned. Evidence on this point was obtained by sterilizing cultures of *Ps. fluorescens* which had grown in manured soil for a period of fourteen days and reinoculating with *B. cereus.* Control flasks of sterile soil were inoculated for comparison. Quantitative analyses were made at daily intervals to determine relative rates of multiplication. The soils in which *Ps. fluorescens* had grown proved to be as favorable for the growth of *B. cereus* as the uninoculated controls. Within 24 hours from the time of inoculation the number of cells had increased from about 30,000 to 1,250,000 per gram.

This result fails to support the theory that the toxic metabolic products exert the same effect in soils as they do when added to artificial culture media. It is, however, in complete harmony

with the experiments on adsorption. It appears improbable that other species of soil microörganisms are more potent in the production of bacterio-toxin than the strain of *Ps. fluorescens* employed in these experiments. The evidence fails to warrant the conclusion that the failure of *B. cereus* to compete successfully with *Ps. fluorescens* in manured soil is due to the toxin produced by the latter. The theory that free bacterio-toxins accumulate in soils in sufficient amounts to inhibit the growth of bacteria seems very doubtful. It appears to be much more probable that difference in nutritive requirements and relative growth rate rather than inhibition due to toxic metabolic products accounts for the difference in number of cells noted by others.

SUMMARY

1. *Pseudomonas fluorescens* produces a thermostable, filterable dialyzable bacterio-toxin which is both inhibitory and bactericidal.

2. The toxin is not specific in its action but is more active against certain species than others. Fungi are more resistant than bacteria. Spore-forming bacteria and micrococci are very sensitive, while colon bacteria are resistant. The substance is not isoinhibitory.

3. The amount of toxin produced depends on the composition of the culture medium and availability of oxygen. Maximum production occurs in cultures grown on the sloped surface of Uschinsky's agar.

4. The toxin persists longer in agar than in broth cultures and resists desiccation for long periods of time. It is soluble in alcohol, and is adsorbed by substances in soil and by charcoal.

5. The toxin is produced in sterilized manured soils inoculated with *Ps. fluorescens*. Alcoholic extracts from such soils are inhibitory for the more sensitive species only.

6. Soils which have supported a vigorous growth of *Ps. fluorescens* in flask cultures are suitable for the growth of *B. cereus* after sterilization.

7. Samples of soil from fields, gardens, pastures and meadows failed to yield toxins by the same methods of extraction employed for the pure cultures. It appears that the toxin studied here is

of a different nature from that of the thermolabile substances reported by others from soils.

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BACTERIAL CALORIMETRY

I. GENERAL CONSIDERATIONS. DESCRIPTION OF DIFFERENTIAL MICROCALORIMETER

STANHOPE BAYNE-JONES

From Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

The production of heat by living bacteria is one of the transformations of energy brought about by these organisms. Although the rise in temperature of fermenting liquids, decomposing manure and other organic substances has been studied on account of its economic effects, and although, periodically, investigations of heat production in relation to the energetics of microörganisms have been made, it is not possible at this time to state definitely the significance of this liberation of heat or to correlate it with the various phases of the growth and activity of bacteria. In fact, the most recent and valuable compilation of this knowledge by Buchanan and Fulmer (1928) contains a paragraph on the utilization of energy in the development of heat which concludes with this sentence: "More complete studies on the utilization and partition of energy in the growth of microorganisms are needed."

The rise in temperature of organic substances undergoing bacterial decomposition has been called "thermogenesis." Its importance is connected chiefly with so-called "spontaneous heating," which is useful in some agricultural processes, but harmful in others, particularly those in which destructive combustions occur. Within the past year, James (1927–1928) has investigated this type of thermogenesis and has published a comprehensive review of the literature on the subject. These studies, however, have been concerned primarily with increase in tem-

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perature and do not afford data on heat production of the kind which can be used in the investigation of energy exchange.

In considering the energy transformations of microörganisms the assumption is made that, when light is excluded, these organisms derive from the aerobic or anaerobic oxidation of foodstuffs all the energy required for their growth, structure formation, maintenance, repair, reproduction, motility, physical states and all the work associated with their vital processes. There is no evidence that they receive energy from other sources. Absorption of heat from the environment affects their chemical and physical reactions, but this heat is not a source of energy to the minute isothermal cell. Hill (1912) has clearly demonstrated the fallacy of regarding bacteria as heat engines, as was suggested by Simonson (1910).

On this assumption, attempts have been made to strike the energy balance of bacterial processes on a calorimetric basis. Rubner (1902, et seq.), as a part of his epochal investigations of the energy transformations of living organisms, measured the heat produced by bacteria. Rubner's bacterial calorimeter was a vacuum flask, carefully insulated and fitted with a delicate thermometer. In order to arrive at an energy balance he determined the heat liberated by the bacteria, the combustion caloric values of the uninoculated medium, the residue medium after growth and the total growth or "crop" of bacteria. In spite of the difficulties and uncertainties of corrections which must be used to compensate for effects produced by changes in the environmental temperature of this type of calorimeter, Rubner obtained important results, which will be discussed later. In a somewhat similar manner, omitting the direct measurement of heat produced, Tangl (1903) and Terroine and his associates (1922, et seq.) have attempted to discover how much energy microörganisms require for the formation of their structure. It does not seem to be probable that combustion values of foodstuffs, residue and bacteria can furnish the information required for the solution of these problems. Among various factors which are not taken into account by this method is the free energy of the substances utilized. The free energy of foodstuffs may differ

considerably from the energy value derived from measurements of the heat produced on combustion. Oppenheimer (1925) has shown that the combustion values of foodstuffs are "practically" equal to their free energies. On the other hand, Baron and Pólányi (1913) and Linhart (1920), and others, have so clearly pointed out that the free energy of these substances must be taken into consideration that it would be unwise to rest modern studies of bacterial calorimetry upon the older values of energy obtained by combustion methods.

The most recent and valuable studies of bacterial calorimetry are those of Meyerhof, A. V. Hill and Shearer. By combined measurements of oxygen consumed and carbon dioxide liberated, together with measurements of heat produced, Meyerhof (1912) has determined the caloric quotient of some phases of bacterial growth. This quotient is expressed as the ratio: heat produced in calories/milligram of oxygen consumed. Through study of this quotient, Meyerhof discovered evidence of the extreme complexity of the factors involved in the production of heat in a culture of bacteria, showing that not all of the heat found in the cultures can be attributed to the oxidation processes of the bacteria. The contribution made by Hill (1911–1912) to the advance of this work has been most direct in the form of the useful differential microcalorimeter devised by him. The great value of his general investigations of the energetics of cellular processes will appear in all phases of this work. Shearer (1921), using Hill's calorimeter, discovered that bacteria liberate much less heat when utilizing free amino acids than when they use more complex protein split products.

It is not intended to review all of this literature here. It will be more advantageous to take up the consideration of appropriate papers in connection with experimental measurements of the heat produced by bacteria at various stages of their growth and activity. In general the literature shows that the physiologists have for a long time perceived the advantage of using bacteria as subjects for the investigation of the energetics of cells. Rubner's belief that the metabolism of bacteria follows the same laws as that of the cells of higher organisms has been justified by the discovery from the study of bacterial processes of laws govern-

ing the metabolism of the cells of higher organisms. For example, recent advances in the knowledge of the mechanism of the contraction of muscle and of the respiration of cancer cells (Warburg, 1927) owe a part of their success to the results of the investigations of the energy exchanges of bacteria.

From the point of view of the bacteriologist, however, some of the investigations of these physiologists seem to suffer from lack of attention to the strictly bacteriological aspects of the subject. Precautions to keep cultures from contamination have not always been adequate, and in some instances have not even been attempted. It is apparent also that, through ignorance or neglect of the phases of growth of microörganisms, some of the most interesting periods of metabolism have not been studied.

It is evident that one of the first requisites for the investigation of these problems is an apparatus by which heat production by bacteria may be measured accurately.

DESCRIPTION OF DIFFERENTIAL MICROCALORIMETER

A calorimeter for use in the measurement of heat production by bacteria should be sensitive to small differences in temperature in the apparatus and as independent as possible of temperature changes in its external environment. It should be so constructed as to permit sterilization of all parts in contact with the culture, should allow stirring of the culture without causing unequal changes in temperature and should be provided with suitable openings, protected against temperature disturbances due to evaporation, for the introduction of the inoculum and for the withdrawal of small samples of fluid and gases. The differential microcalorimeter described by A. V. Hill (1911–1912) is an apparatus suitable for the measurements of small amounts of heat and automatically eliminates errors due to small changes in the environmental temperature. Since my use of this apparatus for the measurement of the heat liberated by reactions of antigens with antibodies (Bayne-Jones, 1925) I have made some changes in it which permit sterilization of the culture flasks and secure protection against contamination. These modifications, together with the methods used in calibrating the apparatus will be described in the following paragraphs.
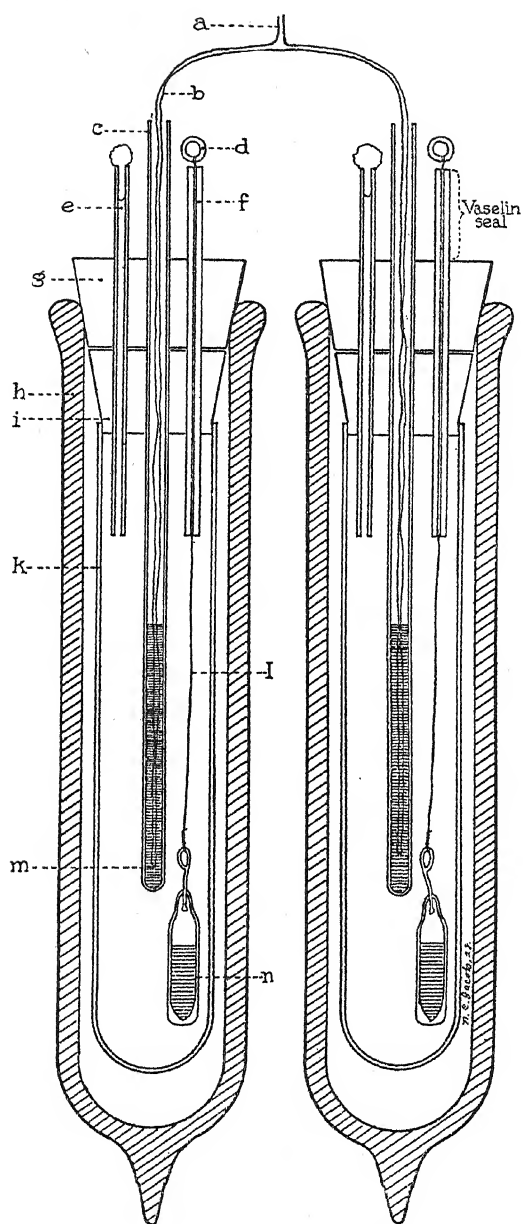
FIG. 1. SECTIONAL VIEW OF DIFFERENTIAL MICROCALORIMETER

The basic parts of the calorimeter are two cylindrical silvered vacuum Dewar flasks, 30 cm. long, 4.5 cm. wide, 380 cc. volume. Figure 1 is a sectional drawing showing these flasks and their contents. The Dewar flasks cannot be sterilized by heat without altering their thermal properties. Therefore, a vessel which can be filled with the requisite amount of medium and sterilized in the autoclave is fitted inside the Dewar flask. This vessel, K, is a rounded bottom, thin-walled pyrex glass tube measuring 20 by 4 cm. Its upper end is closed with a rubber stopper, I, or may be made of glass with suitable tubes fused into it. A second rubber stopper, G, is used to close the Dewar flask. Three glass tubes are passed through these stoppers. The tube marked E has an inside diameter of 3 mm. It is open at both ends, passes into the inner vessel, reaching almost the level of the fluid and has its outer end, 8 cm. above the outside stopper, plugged with cotton. Inoculation of the medium is made through this tube, and through it small samples are withdrawn by capillary pipette. Tube C is a thin-walled glass tube, 4 mm. in diameter, closed at the lower end to serve as a receptacle for the thermocouple. As it is impracticable to sterilize the thermocouples, A and B, they are placed inside this tube after the sterilization of the culture vessel. A small amount of fluid is placed in this closed tube to aid in the conduction of heat to the junctions of the thermocouple. This arrangement makes the temperature measuring system slightly less sensitive, introducing a short lag. These slight errors, however, having values which can be determined, are much less serious than the uncertainties produced by thermocouples contaminated with bacteria. The third tube, F, is a thick-walled capillary tube to provide a sleeve for the thread or fine wire, L, to which the stirring bulb, N, is attached. This bulb is composed of glass partly filled with mercury and is of such a size as to allow it to be moved up and down in the vessel without striking the wall of the thermocouple tube. After sterilization, the upper part of this capillary tube is filled with vaseline to shut out bacteria and prevent evaporation. The ring, D, is attached to the arm of an automobile wind-shield wiper, the oscillations of which raise and lower the bulb, N, stir-

ring the contents of the culture vessel. Bulbs of this type are more satisfactory than air bubbles or rotating blades for stirring the cultures. Air causes frothing of the culture medium, evaporation, temperature disturbances which cannot be controlled and irregular distribution of the bacteria. Rotating blades stir adequately. But it is very difficult to revolve the blades in each flask at the same rate and the mechanism for rotating the blades takes up more room than that used to raise and lower the bulbs.

Two Dewar flasks with sterilizable inner vessels compose the differential calorimeter. One vessel, into which is placed one junction of the thermocouple, contains the culture. The second vessel, serving as a control, contains the other junction of the thermocouple. The pair of vessels is so adjusted that temperature differences between them due to any other causes than the changes occurring in the culture are automatically almost completely balanced out. The temperature changes indicated by deflections of a galvanometer attached to the terminals of the thermocouples are, therefore, directly referable to changes in the culture. In order to protect the calorimeter against temperature disturbances from the outside, as an additional precaution, the flasks are submerged in a water bath, the temperature of which rarely varies more than 0.01°C., and often remains constant for many hours.

In order to balance out temperature changes due to fluctuations in the environmental temperature, the two flasks are given the same coefficient of thermal conductivity by placing an appropriate amount of fluid in each.

The conductivity constant for loss of temperature by a vacuum flask may be calculated from the following expression:

$$K \log e = \frac{1}{t} \log \left(\frac{A - T_0}{T - T_0} \right)$$

where K = conductivity coefficient for temperature loss.

t = time, usually in hours.

A = initial temperature.

T = temperature of fluid in flask at time t.

T_0 = temperature of environment (e.g., water bath).

$\log e = 0.434.$

This coefficient K, varies with the amount of fluid in the flask. Hence K can be made to have any desired value within certain limits by increasing or decreasing the amount of fluid in the flask. After determining K for a standard volume of fluid in one flask, the other flask can be given an identical value of K by putting into it more or less than the standard amount of fluid. This amount of fluid can be determined from tables prepared when the calibrations are made.

Corrections for heat loss, which are used in the final computation of the total heat produced, are based upon the value of this coefficient of temperature loss, K. In order to calculate the total amount of heat produced during an experiment it is necessary to add to the observed heat production an amount equal to that which has been lost by conduction to the outside. The formula for this is given by Hill as follows:

Total heat liberated = (heat capacity of the flask and fluid) $(T - T') - (A - A')$ [K (area of the curve)]

where $T - T'$ = the observed difference in temperature between the two flasks;

$\quad A - A'$ = the initial value of $T - T'$.

$\quad\quad\quad K$ = the coefficient of conductivity for loss of temperature. The value used is taken proportional to the interval of time for which the calculation is to be made.

These values are known from the observations.

The expression K (area of the curve) refers to the curve relating the difference in temperature between the two flasks to the time during which the observations are made. The area of the curve can be obtained with sufficient accuracy for any interval by multiplying the observed value $(T - T')$ in the middle of that interval by the duration of the interval in hours or fractions of hours. Therefore, K (area of the curve) = K [value of $(T - T')$] in the middle of the interval. This expression gives the temperature lost during the particular interval of time.

The loss of heat calculated thus for each interval, added to the loss calculated to the end of the previous interval and finally

added to the observed heat production, gives the total heat liberated.

The temperature changes are measured by means of thermocouples composed of junctions of copper and constantan wires. The constantan wire, B & S. No. 30 is 0.25 mm. in diameter, the copper wire, B. & S. No. 32 measures 0.202 mm. in diameter. These fine wires are silk-covered and are coated with shellac after completion of the junctions. The junctions are made by winding the copper around the constantan wire and fixing it with a small piece of solder. Elements composed of one to 10 junctions have been used. A single pair, or at most three pairs of junctions give sufficient electromotive force for the measurements to be made in this work. As an additional insulation and protection against wetting, the wires and junctions are enclosed in very thin capillary tubes, sealed at the bottom.

The copper wires leading from the thermocouple junctions are covered with rubber tubing and passed through glass tubes, well covered with rubber, to two pools of mercury in glass cups set into a block of wood. These pools of mercury are placed close together and protected as much as possible against unequal heating or cooling, to avoid thermoelectric effects at this point. Copper wires connect the mercury pools to the terminals of the galvanometer. Numerous tests have demonstrated that this system of wiring is free from parasitic currents arising from bimetallic junctions.

The galvanometer is a Type HS reflecting instrument made by the Leeds and Northrup Company. The total resistance of the galvanometer is 17.5 Ω. Its microvolt sensitivity is 4.5 mm. per microvolt at 1 meter and its external critical damping resistance is 50 Ω. The resistance of the galvanometer is approximately equal to the resistance of the thermocouples and circuit.

The readings indicative of temperature changes at the junctions of the thermocouple are taken directly from the scale divisions of the galvanometer deflection at 1 meter. No potentiometer is used. The calibration of the scale divisions in terms of temperature is made by numerous comparisons with Beckmann thermometers in the flasks with the thermocouple junctions.

Most of the thermocouples used give deflections in which 1 scale division = 0.003°C.

No experiment is begun until the pair of flasks composing the calorimeter are in temperature equilibrium with each other and with the water bath in which they are submerged. The Dewar flasks are rarely lifted out of the water bath, but are raised only high enough to allow removal of the inner vessels. After the inner vessels, containing sterilized culture medium, are brought from the autoclave they are placed in a pot of water until their temperature has cooled down to approximately that of the water bath. They are then placed in the Dewar flasks, submerged below the surface of the water bath, and at this time the thermocouples are let down into their appropriate tubes. The stirrers are connected and started. The whole system is left in this condition for ten to eighteen hours, which is the time required for the establishment of temperature equilibrium. At the end of this period, and after at least thirty minutes observation showing zero deflection of the galvanometer, the experiment is begun by the introduction of the inoculum into one of the flasks. With proper precautions, 1 ml. of a fluid culture may be introduced through tube E (fig. 1) by means of a capillary pipette without causing more than a slight change in temperature. Compensation for this is made by a slight shift of the zero of the galvanometer. Aseptic precautions at this time, and at subsequent removals of small amounts of the culture through tube E avoid contamination with undesirable bacteria.

<div align="center">CALIBRATION</div>

<div align="center">*Calibration of flasks*</div>

The determination of the value of the coefficient of temperature loss, K, is made by placing in a flask warm water, 1° to 2°C. higher than that of the surrounding temperature and allowing this to cool. Observations of the temperature at regular intervals of time provide data for the calculation of K. A number of such observations are made and an average value of K is taken from these results. The temperature may be measured with a

Beckmann thermometer or with the thermocouples. When the temperature in the flask is within 1°C. of the temperature of the water bath the cooling curve is in a region in which errors in measurement of the temperature have a larger effect than they

TABLE 1

Determination of K for flask No. 1, containing 100 cc. water

Values of A and T are expressed as the degree of temperature above that of the water bath.

t TIME	T_0 WATER BATH	A INITIAL TEMPERATURE	T TEMPERATURE AFTER INTERVAL	K
hours	°C.	°C.	°C.	
0	37.5	0.6405		
1	37.5	0.6405	0.528	0.1932
2	37.5	0.6405	0.453	0.1729
3	37.5	0.6405		
4	37.5	0.6405	0.333	0.1633
5	37.5	0.6405	0.285	0.1620
6	37.5	0.6405	0.242	0.1626

The value of K for each hour is calculated from the equation:

$$K \log e = \frac{1}{t} \log \left(\frac{A - T_0}{T - T_0} \right)$$

The average value of K per hour from these 5 determinations is 0.1708. Calculations of temperature corrected for loss by cooling are made as follows, using 0.1708 as the value of K per hour. The values for the temperature at the half-hour intervals are taken from the graph.

TIME	TEMPERATURE OBSERVED	$K \times$ VALUE OF MID. ORDINATE	TOTAL TEMPERA- TURE LOSS	CORRECTED TEMPERATURE
hours	°C.			°C.
0	0.6405	0	0	0.6405
1	0.528	0.099	0.099	0.627
2	0.453	0.083	0.182	0.635
3	0.395	0.071	0.253	0.648
4	0.333	0.061	0.314	0.647
5	0.285	0.053	0.367	0.652
6	0.242	0.045	0.412	0.654

do in cases in which the temperature difference is greater. Nevertheless, the equation for the calculation of the coefficient of temperature loss holds. An illustration of the method of determining K and of the calculations for corrections for loss of temperature is

given in table 1 and the accompanying graph, figure 2. The values of the corrected temperature lie on an approximately straight line parallel to the time axis at the level of the value of the initial temperature.
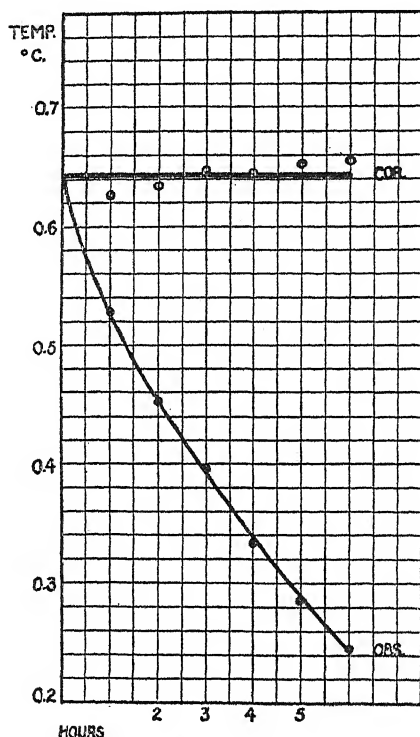
FIG. 2. GRAPH OF COOLING CURVE OF CYLINDER AND INNER VESSEL

Determination of sensitivity and accuracy

By liberating a small known amount of heat from a resistance coil in the fluid in one of the flasks it is possible to determine the sensitivity and lag of the system and to evaluate the errors in the measurements. A small coil of nichrome wire is placed in the fluid in one of the pair of differential flasks and a measured electric current is passed through it for a definite period of time.

A short lag occurs, but measurable changes in temperature are

detectable within a minute after the start of the flow of current. This interval is of no significance in the kind of work for which the apparatus is intended.

The heat produced by the coil is calculated from the formula:

$$H = \frac{RI^2}{4.18} \text{ gram cal. per sec.}$$

where H = heat produced.

   R = resistance in ohms.

   I = current in amperes.

   4.18 = factor for converting joules into gram calories.

In one experiment the resistance of the coil was 11 Ω and the current 0.025 amperes. This produced 5.91 gram calories per hour. The corrected temperature at the end of five hours was 0.249°C. The heat capacity of the flask and fluid was equivalent to that of 125 cc. of water. Hence the observed heat production was 6.02 gram calories per hour. The error in this case was +1.88 per cent. In another experiment, lasting six hours, the observed heat production with the same coil and a current of 0.05 amperes was 22.89 gram calories per hour. The calculated heat production amounted to 23.68 gram calories. The error in this case was 3.33 per cent. These experiments demonstrate that the calorimeter and measuring devices are sufficiently sensitive and accurate to measure the production of a small amount of heat, of the order of magnitude of that produced by cultures of microörganisms, with an error of approximately ±3.0 per cent.

Experiments on the measurement of the heat produced by mixtures of definite volumes of absolute ethyl alcohol and water in the calorimeter gave results which differed only within this range of error from the heat production expected by calculation.

The heat capacity of the flask and its contents can be approximated from computations based upon the amount and kind of fluid in the flask, the specific heat of this, and the glass and other materials composing the vessels and apparatus in them. This estimate can be verified and corrected by calibrations in which a known amount of heat is liberated in the flasks. In general, the

heat capacity of the flasks and fluids used has been in the neighborhood of 125, e.g. equivalent to the heat capacity of 125 cc. of water.

As the methods of observation and computation which have been used in the experiments upon the heat produced by bacteria are applied in the case in which a known amount of heat is

TABLE 2

Calibration experiment

Dewar cylinder and vessel No. 1.

100 cc. water in vessel. Heat capacity of flask and contents = 125.

Coefficient of loss of temperature, K, = 0.1780 per hour.

Coil, resistance = 11 Ω.

Current 0.050 amperes. Gram. cal. per hour (calc.) = 23.68.

Thermocouple No. 3 gives 1 scale div. = 0.003°C.

Water bath temperature 37.5° ± 0.01°C.

HOURS	GALV. S.D.	°C. OB-SERVED	$K \times$ VAL. MID. ORD.	TOTAL TEMPER-ATURE LOSS	°C. COR-RECTED	HEAT GRAM CAL. OBS.	HEAT GRAM CAL. CALC.	DIF. GRAM CAL.	ERROR PER CENT
0	0	0	0	0	0	0	0	0	0
0.5	32	0.096							
1	58.8	0.1764	0.017	0.017	0.1934	24.22	23.68	0.54	+2.28
1.5	82	0.246							
2	105	0.315	0.0438	0.0608	0.3758	46.97	47.36	0.39	−0.82
2.5	127	0.381							
3	144.5	0.4335	0.0678	0.1286	0.5621	70.26	71.04	0.78	−1.09
3.5	163	0.489							
4	180	0.540	0.087	0.2156	0.7556	94.44	94.72	0.28	−0.29
4.5	194	0.582							
5	209	0.627	0.1035	0.3191	0.9461	117.26	118.40	1.14	−0.96
5.5	225	0.675							
6	235	0.705	0.120	0.4391	1.441	143.01	142.08	0.93	+0.65

liberated in the calorimeter, one of the calibration experiments is given in detail in table 2.

The curve of temperature changes which occurred in this experiment is shown in the accompanying graph, figure 3, in which temperature is plotted against time. The temperature, corrected for heat loss, falls on a straight line, ascending regularly, which is to be expected as the result of the continuous liberation of a constant amount of heat in the calorimeter. It is additional

evidence of the validity of Hill's formula for the calculation of the coefficient of temperature loss and shows the correctness of the value calculated for this case.
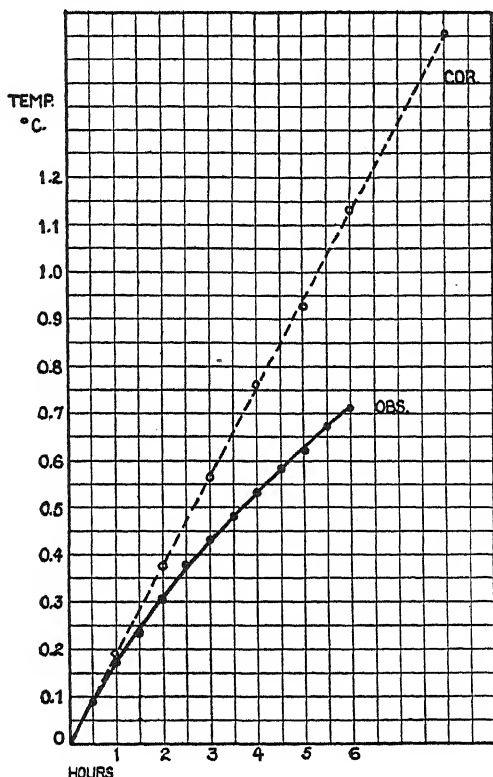
FIG. 3. GRAPH OF OBSERVED TEMPERATURE CHANGES PRODUCED BY HEATING COIL IN ONE VESSEL OF CALORIMETER

The corrected curve is a straight line, showing liberation of a constant amount of heat.

As an additional check on the ability of the calorimeter to eliminate automatically or balance out temperature changes due to warming or cooling the environment of both flasks, a pair of flasks, adjusted to have the same value of the coefficient of temperature loss, K, have been placed close together and left in posi-

tion for forty-eight and ninety-six hours. During this time, the temperature of the room or water bath rose and fell during intervals of two to ten hours through a range of about 2°C. The temperature difference within the flasks during this period, as measured by the thermocouple, varied between 0 and 0.012°C. These changes took place very slowly. Small rapid fluctuations produce no detectable temperature differences between a pair of balanced flasks.

This calorimeter has been used to measure the heat production of bacteria in several types of culture media. Most of these cultures have produced a rise in temperature of 0.2 to 0.5°C. in eight to ten hours, liberating an amount of heat easily measured by the calorimeter, and the recording system. As the curves of heat production require detailed analyses, they will be described and discussed in the subsequent papers of this series.

SUMMARY

This paper describes modifications of the differential microcalorimeter of A. V. Hill which adapts it to the study of heat produced by growing bacteria. The chief changes are the addition of an inner vessel which can be filled with the required amount of culture fluid and sterilized. Other minor changes permit the insertion of the thermocouples without contaminating the media, mechanical stirring in place of stirring by air bubbles, and a tube for the introduction of the inoculum and the withdrawal of small samples.

Data obtained from calibrations are presented showing that the apparatus is sufficiently sensitive and accurate to measure the production of approximately 6 gram calories per hour with an error of ±1.88 per cent and the liberation of 23.68 gram calories per hour with an error of ±3.33 per cent in a vessel and fluid having a heat capacity equivalent to 125 cc. of water. Heat production by cultures of bacteria is within these limits in most of the media employed and it is estimated that the error in the measurements within this range is approximately ±3 per cent.

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BACTERIAL CALORIMETRY

II. RELATIONSHIP OF HEAT PRODUCTION TO PHASES OF GROWTH OF BACTERIA

STANHOPE BAYNE-JONES AND HENRIETTA S. RHEES

From Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

Realizing the importance of establishing the time-relationships of a biological process, we have begun our investigations of bacterial calorimetry with experiments to determine the course of the production of heat during the growth of a culture of bacteria. At the points where the temperature was recorded we have counted the bacteria in the culture. From the results of these studies, which will be reported in this paper, we have been able to plot the curve of heat production with respect to time, discovering points of inflection of this curve, and have correlated the curves of heat production and of growth of pure cultures of several varieties of bacteria in different kinds of media. Quantitative data were secured as to the number of bacteria present at each unit of time and the gram calories liberated by them. Measurements of the sizes of the bacteria, determinations of their surface area and chemical studies of their metabolism were not made at this time as it seemed wiser to postpone these for future investigations which will be facilitated by being based upon a knowledge of the relationship of the production of heat to the phases of the growth of microörganisms.

Little definite information on this subject could be gathered from the literature, as the physiologists who have done most of the work in this field have overlooked a number of the significant phases of bacterial growth. Curves of heat production with respect to time appear in many papers. But in most cases the

long intervals of time between the observations passed over periods when the culture was undergoing great changes and in no papers have we found definite statements of the age and amount of the culture used as the inoculum or counts of the bacteria present at the moments when the temperature was noted. In view of the fact that genuine interest in the growth curves of microörganisms is comparatively recent, we would not expect to find attention paid to these points in the older literature of calorimetry. Among such papers, in which the course of heat production of cultures was noted without a simultaneous accumulation of enough bacteriological data to permit their analysis, may be placed those of Rubner (1911) and A. V. Hill (1911–1912). The graphs of heat production in souring milk, presented in Hill's paper, show that after a latent period of nine to ten hours, heat was produced at a rapid rate during the next four hours and thereafter at a reduced rate. In connection with Shearer's observations and our own, this change of the slope of the curve of heat production between the third and fourth hours after the beginning of active growth of a culture appears to indicate a definite metabolic change at that time. Shearer (1921), in a very interesting account of the way in which *B. coli* builds up amino acids into its protoplasm with relatively little waste of energy in the form of heat, presented a number of curves of heat production with reference to time. These curves show a short period of lag, with subsequent changes in the rate of liberation of heat at the third, sixth and twentieth hours. As Shearer did not attempt to determine the number of bacteria present at these points, it is impossible to refer his curves to actual growth curves. Our results have been quite similar, in general, to those obtained by Shearer, lending additional support to the assumption that these points of change in the slope of the curve are in some way definitely related to changes in the metabolism of the bacteria.

APPARATUS

The calorimeter used in these experiments was a modified form of the differential microcalorimeter devised by A. V. Hill (1911–1912). The modifications, thermocouples, galvanometer and

method of making measurements have been described in the first paper of this series (Bayne-Jones, 1929). The changes made in Hill's apparatus permit sterilization of the culture vessels and aid in avoiding contamination during the process of inoculation and withdrawal of samples. The error in the measurements was estimated to be ±3 per cent.

ENUMERATION OF BACTERIA

It is unnecessary to review all the recent literature on the subject of methods of counting bacteria. Our experience with plate-counts was unfavorable to the use of that method in connection with this work. For our observations, which extend over periods of eight to ten hours, we found that direct counting of bacteria in a Helber chamber 0.01 mm. deep, using a diluent containing basic fuchsin, together with an opacimetric determination upon a duplicate sample according to the method of Gates (1920) gave us reliable data from which growth curves could be constructed. It is admitted that direct counts and opacity determinations do not distinguish between living and dead bacteria, unless a special staining technic is used, such as that devised by Henrici (1923). On the other hand, it is well known that more than 90 per cent of bacteria in cultures less than twelve hours old are living and it may be presumed that nearly all the organisms in such a culture have some degree of vitality. In fact, Jensen (1928) regards the growth curves constructed from data obtained with his special method of direct counting as more representative of natural conditions than those based on the results of plate counts. Organisms in old cultures, occurring in clumps, and flakily growing organisms such as *B. subtilis* could not be counted by any method.

The small samples of the culture required for these counts were withdrawn by a sterile capillary pipette through a small tube passing through the rubber stoppers of the calorimeter vessel. Numerous tests demonstrated that no appreciable disturbance of the temperature of the culture was produced by this procedure.

The inoculum was introduced through this same tube at the beginning of the experiment. This usually consisted of 1 cc. of a twelve- to eighteen-hour broth culture of an organism. The culture was transplanted daily to reduce the period of lag following inoculation of the medium in the calorimeter. In these experiments, the period of lag was always less than one hour, and, in fact, evidences of heat production were often apparent fifteen minutes after the inoculation. Care was taken at all stages to ascertain by smears and plates that the material used for inoculation and the growth in the flask of the calorimeter were pure cultures of the organism in use. Whenever contamination occurred, the experiment was discarded.

As has been explained in the first paper, the medium in the calorimeter flask was always sterilized in the autoclave at least ten hours before the commencement of an experiment, and the medium and the calorimeter in the water bath were allowed to reach an equilibrium of temperature. While we attempted to have the amount of broth culture used for the inoculum at the same temperature as the medium in the calorimeter, we often failed to do this. On this account, differences of 0.003 to 0.01°C. were produced by the introduction of the inoculum. These small differences at the start of the experiment were usually discounted by a shift of the zero of the galvanometer attached to the thermocouples, and in any case had little or no influence upon the subsequent course of heat production by the organism.

With the exception of a few experiments made in 1926, the cultures were not aerated, but received oxygen through the small plugged tube passing through the rubber stoppers of the calorimeter. The condition within the culture flask was presumably slightly less aerobic than that in ordinary culture tubes plugged with cotton. At this time we did not attempt to study the gaseous exchanges of the organisms or the effect of oxygen tension upon heat production.

The temperature of the environment, which may be called the incubation temperature, was 37.5 ± 0.01°C. During most of the

experiments, the fluctuations of the temperature of the water bath were less than 0.01°C., usually within 0.005°C. as measured by a Beckmann thermometer.

In the first paper of this series, the calculations used to determine the coefficient (k) of loss of temperature of the calorimeter flask have been set forth and experiments with heating coils have been recorded in detail to show how this factor was used in correcting the temperature. For most of the vessels, this coefficient was approximately 0.1780 per hour. In most cases, 100 cc. of culture medium were placed in the flask, which with its liquid and glass contents had a total heat capacity equivalent to 125 cc. of water. The corrected temperature multiplied by the heat capacity of the flask and contents gave the total gram calories of heat produced.

The heat produced by each bacterium was obtained directly by dividing the total gram calories by the number of bacteria present in the 100 cc. of culture medium. This value, of course, was actually too small to be measured, but some interesting relationships were discovered through the graphic use of it.

The same fact could be reached by a calculation. We are indebted to Dr. R. E. Buchanan for the formula for this calculation, having found it first in a note on the "Determination of the fermentation capacity of a single bacterial cell," published by him in Abstracts of Bacteriology in 1918. As a number of errors occur in that report, it seems advisable to present here the verified development of the correct formula which Dr. Buchanan sent us. The assumptions upon which the calculations are based are that the organisms are multiplying in geometric progression at a definite rate, and that each organism is excreting the substance, (or producing heat), at a definite uniform rate. The amount of the substance (or heat) produced must be measured at the end of each unit of time, and the number of bacteria counted at the beginning and at the end of each unit of time.

Let B = number of bacteria at beginning.

b = number of bacteria after time t.

n = number of generations in time t.

g = average generation time.

m = amount of substance (or amount of heat) produced per cell per unit of time.

S = total amount of substance (or heat) produced in time t.

Then

$$n = \frac{t}{g}$$

$$b = B\ 2^n = B\ 2^{\frac{t}{g}}$$

The amount of substance (or heat) produced during any instant dt would be $m \cdot b \cdot dt$. And the total amount (S) produced in time t would be

$$S = \int_0^t m \cdot b \cdot dt$$

Substituting

$$S = mB \int_0^t 2^{\frac{t}{g}}\ dt$$

upon integration

$$S = \frac{g\ m\ B \left(2^{\frac{t}{g}} - 1 \right)}{\ln 2}$$

Substituting:

$$S = \frac{Mt\ (b - B)}{\ln \dfrac{b}{B}}$$

$$M = \frac{S\ \ln 2}{g\ (b - B)} = \frac{S\ \ln \dfrac{b}{B}}{t\ (b - B)}$$

Whence

$$M = \frac{S\ 2.303\ \log \dfrac{b}{B}}{t\ (b - B)}$$

From the last expression, we have calculated the amount of heat produced by a single bacterium during each hour of the growth of the culture.

In accordance with the assumptions upon which the formula rests, the values obtained during the logarithmic period were most significant.

During the past two years, we have made a considerable number of experiments. The results of these have been in general so similar that several of them may be selected with propriety to illustrate the general principles with which this report is concerned. As conditions of media, size of inoculum, age of culture and other factors vary more or less in each experiment, a false conception of the relationship of the heat production to growth is given by an average of all results. Indeed, each individual experiment has a distinct validity. Nevertheless, we have averaged the results of six experiments with *B. coli*, which were almost identical, and will present this composite curve for comparison with the data of individual cases. Four cases will be presented as follows: heat production by (1) *B. coli* in a 2 per cent peptone broth, average of 6 experiments, (2) *B. coli* in 2 per cent Difco peptone water, (3) *B. coli* in 2 per cent Difco peptone water containing 1 per cent glucose and (4) *Staphylococcus aureus* in 2 per cent Difco peptone water.

All temperatures noted in the tables are expressed in terms of degrees Centigrade above that of the water bath. (See tables 1 to 4.)

TABLE 1

Heat production by B. coli in broth (average, plate 1)

Three hundred cubic centimeters of a broth containing 2 per cent Difco peptone— pH = 7.6.

Heat capacity of flask and fluid = 330.

Coefficient of temperature loss (K) = 0.1280 per hour.

Stirring by air saturated with water vapor.

Temperature of incubator = 37.5 ±0.1°C.

Inoculum = 1 cc. of eighteen-hour culture of *B. coli* (strain S) in this broth.

This table is composed of the average results of six experiments.

These results are shown graphically in figure 1, A, B, C and D.

TIME	TEMPERA-TURE CORRECTED	NUMBER OF BACTERIA PER CUBIC CENTI-METER $\times 10^6$	LOG NUMBER BACTERIA PER CUBIC CENTI-METER	TOTAL HEAT GRAM CALORIES OBSERVED	TOTAL NUMBER BACTERIA $\times 10^6$	GRAM CALORIES PER BACTERIA OBSERVED $\times 10^{-9}$	GRAM CALORIES PER BACTERIA CALCULATED $\times 10^{-9}$
hours	°C.						
0	0	1.2	6.07918	0	360.0	0	0
1	0.0045	1.4	6.14613	1.485	420.0	3.54	4.608
2	0.0536	7.2	6.85733	17.68	2160.0	8.182	8.77
3	0.1524	32.0	7.50515	50.29	9600.0	5.24	5.95
4	0.2323	112.0	8.04922	76.65	33600.0	2.28	2.65
5	0.2810	320.0	8.50515	92.73	96000.0	0.966	1.08
6	0.3291	430.0	8.63347	108.60	129000.0	0.842	0.829
7	0.3683			121.53			
8	0.4281			141.27			
9	0.4408			145.46			
10	0.4749			156.71			

TABLE 2

Heat production by B. coli in 2 per cent Difco peptone broth (plate 2)

One hundred cubic centimeters of peptone water containing 2 per cent Difco peptone and 0.5 per cent NaCl—pH = 7.6.

Heat capacity of flask and fluid = 125.

$K = 0.1780$ per hour.

Stirring by moving bulbs.   Culture not aerated.

Inoculum = 1 cc. of eighteen-hour culture of *B. coli* (strain *S*) in the same medium.

Temperature of water bath = $37.5 \pm 0.01°C$.

The results of this experiment are shown in the table and in the curves in figure 2.

TIME	TEMPERATURE		NUMBER BACTERIA PER CUBIC CENTI-METER $\times 10^6$	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	GRAM CALORIES PER BACTERIA OBSERVED $\times 10^{-9}$	GRAM CALORIES PER BACTERIA CALCULATED $\times 10^{-9}$
	Observed	Corrected.					
hours	°*C.*	°*C.*					
0	0	0	8.5	6.9294	0	0	0
1	0.009	0.0095	20.5	7.3117	1.19	0.581	0.491
2	0.0645	0.0716	45.4	7.6570	8.95	1.98	2.02
3	0.1065	0.1309	126.0	8.1003	16.3	1.295	1.25
4	0.123	0.1676	245.0	8.3891	20.9	0.855	0.742
5	0.1395	0.2075	330.0	8.5185	25.9	0.788	0.589
6	0.1545	0.2486	400.0	8.6020	31.0	0.776	0.51
7	0.1665	0.2891	480.0	8.6812	36.1	0.753	0.442
8	0.171	0.324	500.0	8.6989	40.5	0.736	0.235
9	0.1785	0.3627			45.2		
10	0.183	0.399			49.9		

TABLE 3

Heat production by B. coli in 1 per cent glucose (plate 3)

One hundred cubic centimeters of 2 per cent Difco peptone water, with 0.5 per cent of NaCl and 1 per cent glucose — pH = 7.6.

Heat capacity of flask and fluid = 125.

K = 0.1780 per hour.

Stirring by moving glass bulbs.   Culture not aerated.

Inoculum = 1 cc. of eighteen-hour culture of *B. coli* (strain *S*) in this medium.

Temperature of water bath = 37.5 ±0.01°C.

The results of this experiment are shown in the table and in the curves in figure 3.

TIME	TEMPERATURE		NUMBER BACTERIA PER CUBIC CENTI-METER $\times 10^6$	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	GRAM CALORIES PER BACTERIA OBSERVED $\times 10^{-9}$	GRAM CALORIES PER BACTERIA CALCULATED $\times 10^{-9}$
	Observed	Corrected.					
hours	°C.	°C.					
0	0	0	8.64	6.9375	0	0	0
1	0.0375	0.0391	13.28	7.1232	4.88	3.68	4.5
2	0.114	0.1247	42.0	7.6232	15.57	3.71	3.7
3	0.198	0.2402	316.0	8.4996	30.0	0.95	1.17
4	0.246	0.3288	360.0	8.5563	41.05	1.14	1.087
5	0.279	0.4085			51.02		
6	0.294	0.4746			59.15		
7	0.303	0.5371			67.13		

TABLE 4

Heat production by Staph. aureus in 2 per cent Difco peptone water (plate 4)

One hundred cubic centimeters of 2 per cent Difco peptone water containing 0.5 per cent NaCl—pH = 7.6.

Heat capacity of flask and fluid = 125.

$K = 0.1780$ per hour.

Stirring by moving glass bulbs.   Culture not aerated.

Inoculum = 1 cc. of eighteen hour culture of *Staph. aureus* in the same medium.

Temperature of water bath = 37.5 ±0.01°C.

The results of this experiment are shown in this table and in the curves in figure 4.

TIME	TEMPERATURE		NUMBER BACTERIA PER CUBIC CENTI- METER × 10⁶	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	GRAM CALORIES PER BACTERIA OBSERVED × 10⁻⁹	GRAM CALORIES PER BACTERIA CALCULATED × 10⁻⁹
	Observed	Corrected.					
hours	°*C.*	°*C.*					
0	0	0	6.62	6.8208	0	0	0
1	0.015	0.0157	24.0	7.3802	1.96	0.817	1.452
2	0.0564	0.0626	77.0	7.8864	7.82	1.01	1.365
3	0.0939	0.1112	159.0	8.2014	13.9	0.874	0.965
4	0.120	0.1572	293.0	8.4668	19.7	0.673	0.653
5	0.144	0.205	362.0	8.5587	25.6	0.77	0.583
6	0.159	0.246	378.0	8.5774	30.7	0.81	0.559
7	0.177	0.295	426.5	8.6299	36.8	0.863	0.522
8	0.1845	0.336	547.0	8.7379	41.9	0.766	
9	0.195	0.380	656.0	8.8169	47.5	0.724	
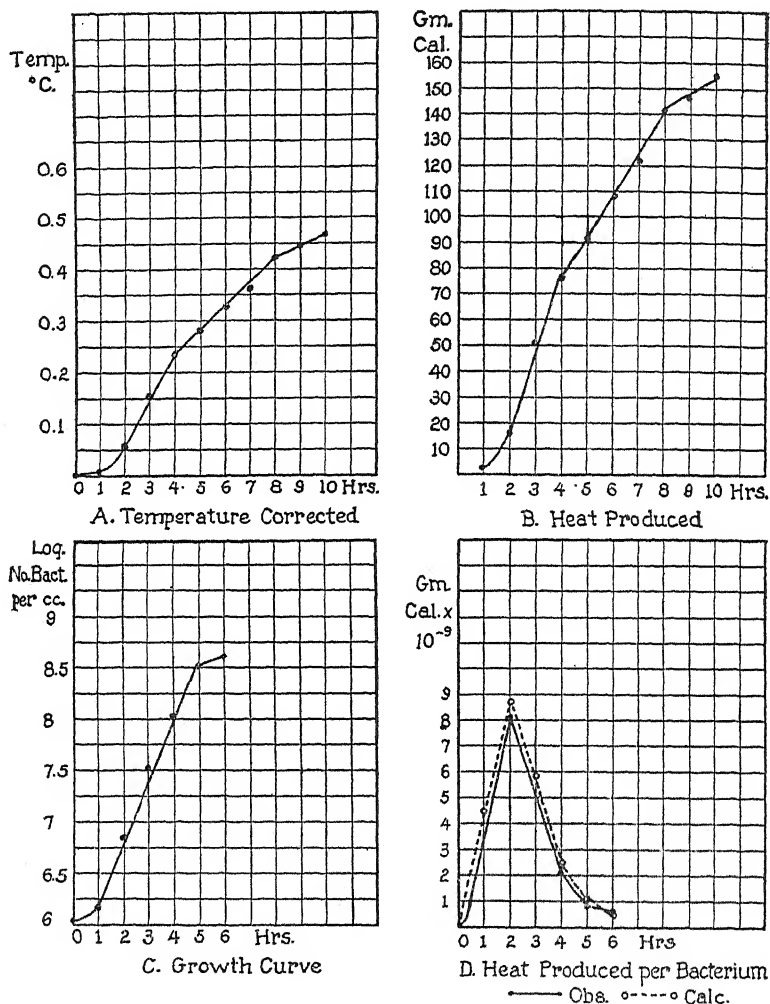
FIG. 1. GRAPHIC REPRESENTATION OF DATA IN TABLE 1

B. coli in peptone broth, aerated. Average of six experiments. Each figure (figs. 1–4) contains four panels showing curves of temperature, total heat production, number of bacteria per cubic centimeter and the heat produced per bacterium at intervals of one hour during the duration of the experiment.
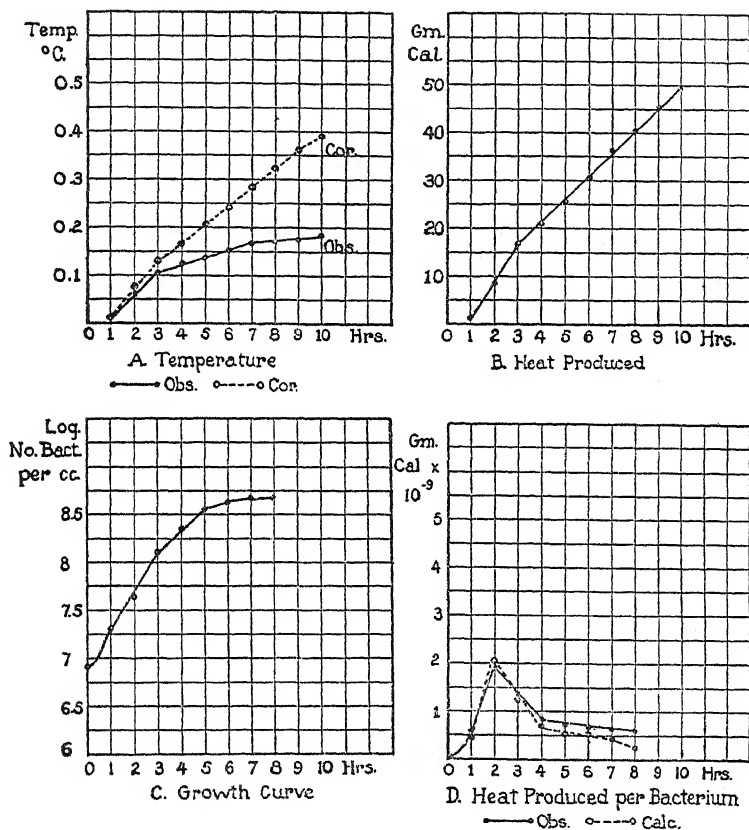
FIG. 2. GRAPHS OF DATA IN TABLE 2

B. coli in 2 per cent peptone water

A. Temperature

B. Heat Produced

C. Growth Curve

D. Heat Produced per Bacterium
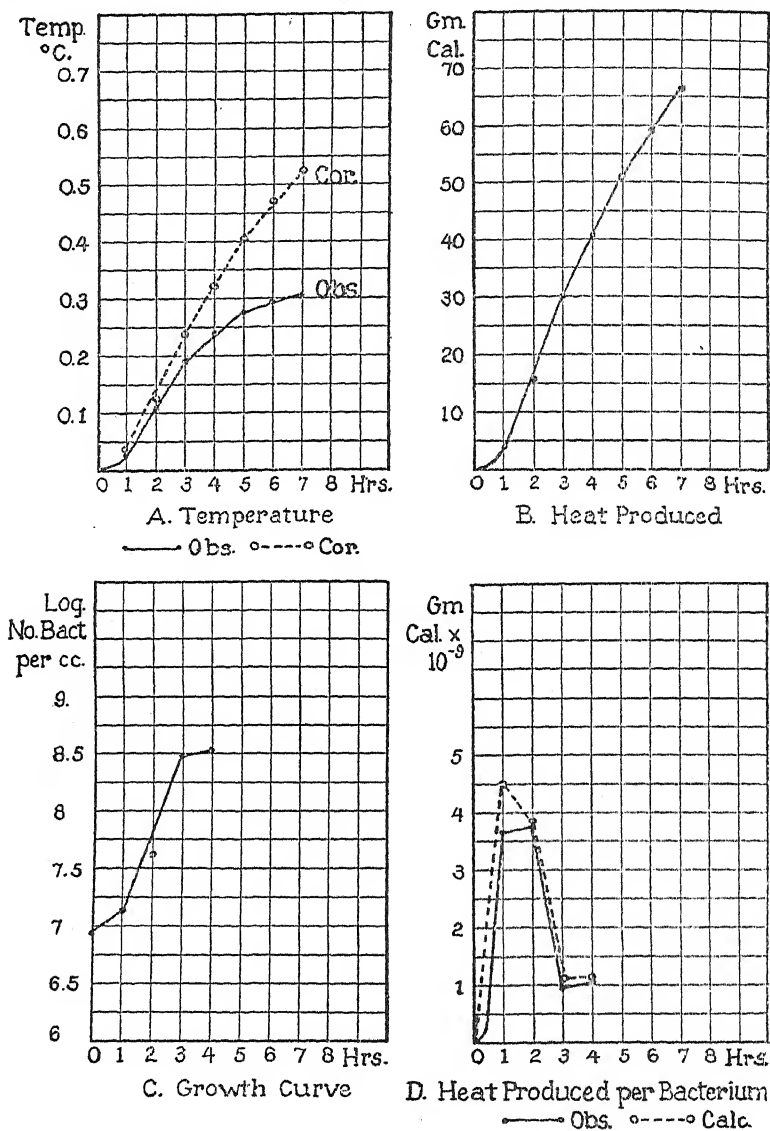
Fig. 3. Graphs of Data in Table 3

B. coli in 2 per cent peptone water with addition of 1 per cent glucose
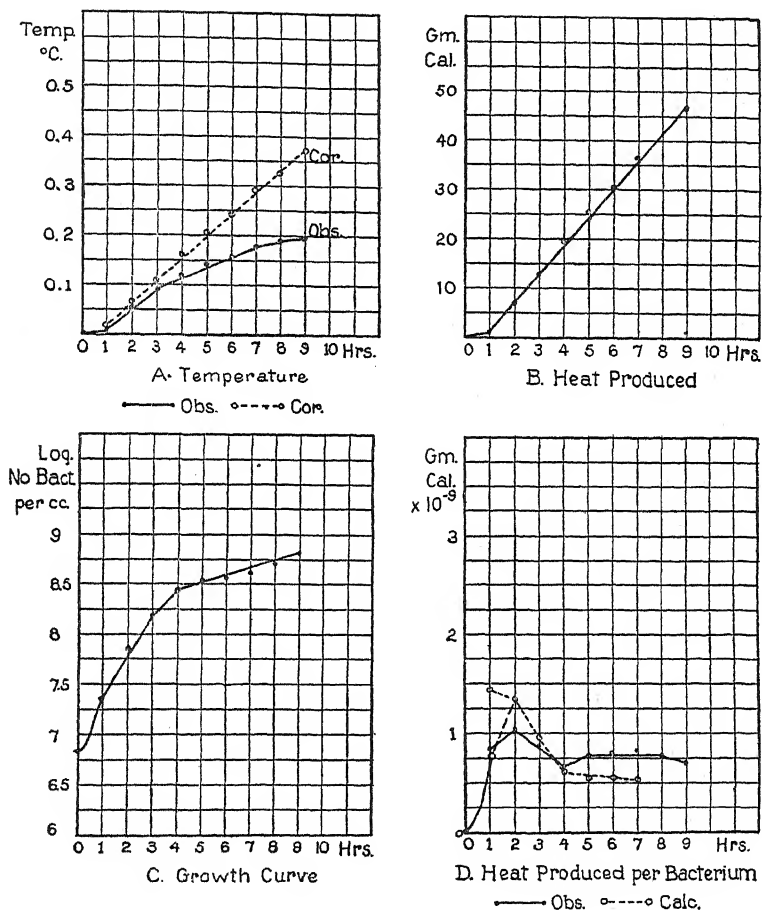
*FIG. 4. GRAPHS OF DATA IN TABLE 4.

Staphylococcus aureus in 2 per cent peptone water

DISCUSSION

Without a full knowledge of all the factors it is obviously fruitless to seek an explanation of the shape of the curve of heat production by growing bacteria. We know in general that this heat has its ultimate source in the oxidation of foodstuffs by the microörganisms. But we do not know at present how much of this heat is directly referable to cellular metabolic processes, and how much is produced by physical processes of motility, changes in surface relationships and the heats of dilution of substances together with the heats of neutralization of acid or basic by-products. As bacteria grow in the midst of the food which they are using and the products which they are excreting, they evidently must give rise to a very complex interacting system of endothermic and exothermic reactions.

It is noteworthy, however, that heat production by cultures of bacteria is an orderly process and that Buchanan's formula for calculating the fermentation capacity of a single bacterial cell fits the curves in those instances of heat production which we have studied. The use of this equation is of great assistance in checking results and in reaching a decision as to the significance of unexpected values. At this time, we do not wish to give the impression that the coincidence of the results of the experiments with those calculated by this formula signify that the assumptions on which the formula were based are correct. It suggests, however, that during the logarithmic period of growth, each cell is liberating heat at a uniform rate.

One conspicuous feature of the observed as well as the calculated heat production is the relatively large amount of heat produced by a young bacterial cell, during the first three hours of growth, as compared with older cells, after the fourth hour of growth. Young cells produced several times as much heat per cell as older ones. During this period the cells are multiplying rapidly, and are large, as Henrici (1923, 1925) has shown. In fact, the shape of the curve during this period is almost identical with that of the index number of the area-length ratio plotted by Henrici, and is of the same shape as, but opposite in sign to, the curve of the index number of the average length. These

agreements still further relate morphological, functional and metabolic states.

In the tables of measurements and in curves drawn to a larger scale than those illustrating this paper, changes in the rate of heat production are discernible corresponding in general to the periods of lag, logarithmic phase, phase of negative acceleration, maximum stationary phase, and phase of decrease. Similar changes in curves of acid production have been noted. Changes in the rate of heat production have occurred invariably at about the end of the first hour, between the third and fourth hour and at about the fifth hour in these rapidly growing cultures. Similar sudden changes in the slope of the time: potential curves of several organisms were found by Clark (1926) and his associates during their studies on oxidation and reduction. These are undoubtedly critical points of fundamental significance in the metabolism of microörganisms, though we are unable to do more than locate them at present.

The studies of cytomorphosis of bacteria by Henrici (1925) and Clark (1928) show clearly that older bacteria are smaller than younger ones, change very little in shape in a short period, and, of course, almost cease to reproduce. This is a fairly long, if indefinite period. These studies of heat production indicate that a period of greatly reduced metabolic activity on the part of each bacterium begins at about the fourth or fifth hour and is maintained at a low constant rate for ten hours, and probably for a longer time. The curves in the illustrations of this paper clearly show this prolonged constant heat production per cell. It is suggested that this phase of heat production may be regarded as a basic metabolic rate of bacteria, if subsequent studies give results which justify the use of such a term as basic metabolism in connection with bacteria.

SUMMARY AND CONCLUSIONS

By means of simultaneous observations of the heat production by bacteria and enumeration of the organisms in the culture it is possible to correlate growth curves with curves of the liberation of heat. A combination of such observations, with those of the morphological changes and life-phases of bacteria, yield some

information as to the metabolic activities of a culture at various periods. By the use of Buchanan's formula, the rate of heat production by a single bacterial cell can be calculated, and the observed values agree closely with those derived by this calculation.

Changes in the rate of heat production are related to changes in the growth rate of bacteria.

Young bacterial cells produce more heat per cell than older ones.

After about the fifth hour of growth, the production of heat reaches a low level and remains constant, suggesting the existence of a basal metabolic rate during that period.

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NOMENCLATURE OF THE ROOT-NODULE BACTERIA OF THE LEGUMINOSAE

I. L. BALDWIN AND E. B. FRED

University of Wisconsin, Madison, Wisconsin

In Bergey's Manual of Determinative Bacteriology, second edition, 1925, the root-nodule bacteria of the Leguminosae are classified in the genus *Rhizobium*, and two species *Rh. leguminosarum* and *Rh. radicicola* are recognized. Differentiation between the two species is established upon the basis of morphology, with particular reference to flagellation, cultural characters, and habitat of the organisms. Only incomplete descriptions of the organisms are given, which fact renders an accurate classification and identification difficult. With the exception of the habitat, no characteristic is described in sufficient detail to render identification possible, and only a very limited number of host plants are mentioned under each bacterial species.

In the seventh edition of Lehmann and Neumann's Bakteriologische Diagnostik, 1927, the nodule bacteria of the various leguminous plants are classified as one species, *Bacterium radicicola* Beijerinck. The possibility of recognizing several subspecies or varieties is mentioned.

At present both American and foreign investigators use several different names for the same organism. Scientific workers have not agreed, either as to the number of species which should be recognized, or as to the terminology which should be applied to them. To avoid confusion many have abandoned the use of scientific terminology and have substituted such terms as "root-nodule bacteria of legumes" or, when a more specific designation was desired, such terms as "alfalfa-nodule bacteria," "soybean-nodule bacteria" or "alfalfa bacteria," "soybean bacteria." At

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best this can be regarded only as a temporary expedient and not as a solution of the question.

Certain other investigators have used the scientific terminology, e.g., *Rhizobium leguminosarum*, *Rh. radicicola*, *Bacillus radicicola*, or *Pseudomonus radicicola* without specifying the plant species from which the organism was secured. The inclusion of organisms differing as widely in their morphology and physiology as the root nodule organisms from alfalfa and bean in a single species *Rhizobium leguminosarum*, as is done by Bergey, leads to confusion. This is particularly true in such reports on the physiology of these microörganisms as that of Werkman, 1927.

The evidence in favor of the use of *Rhizobium* Frank as a generic term for this group seems quite conclusive to the writers. Since Buchanan (1926) has recently given an excellent review of the literature dealing with the synonymy of this group and has presented the facts regarding the use of this term, it is unnecessary to cover this material again, *Rhizobium* was included in the list of names recommended by the Committee of the Society of American Bacteriologists on Characterization and Classification for adoption by the Society; Bergey has adopted the term in his Manual of Determinative Bacteriology; and it has been commonly used by many scientists.

The genus *Rhizobium* was placed in the Family *Nitrobacteriaceae* and the Tribe *Azotobactereae* by the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacteria, and by Bergey in his Manual of Determinative Bacteriology. Löhnis and Hansen, 1921, and many others have criticized this classification of the root nodule bacteria, and have insisted that the organisms are closely related to the colon-aerogenes and the colon-typhoid groups. In the opinion of the authors the view of Löhnis is the more nearly correct one. Certainly the root nodule organisms of the Leguminosae are very similar to certain of the plant pathogens in their morphology, physiology and cultural characteristics: e.g., *Phytomonas tumefaciens*, of the family *Bacteriaceae* and the tribe *Erwineae*. Also, the genus *Rhizobium* has very little in common with the autotropic bacteria of the family *Nitrobacteriaceae*.

The writers suggest that it would be advisable to transfer the genus *Rhizobium* from the *Nitrobacteriaceae* to the *Bacteriaceae* and place it in a new tribe standing close to the *Erwineae* and the *Bactereae*.   *Hyphoidees* was suggested by Vuillemin, in 1905, since it drew attention to the "false hyphae" or "infection threads" formed in the nodule.   Dangeard, 1926, used the term in his classification as a tribal designation.   *Hyphoideae* would thus appear to be the proper designation for the tribe containing the genus *Rhizobium* and it is considered advisable to place this tribe in the family *Bacteriaceae*.

The early students in this field were for the most part of the opinion that all the organisms producing nodules on the roots of leguminous plants should be considered as a single species, although many investigators recognized that certain important differences were apparent among the representatives from the different plant species.

Frank, in 1879, noted certain differences between *Lupinus* and *Lathyrus* as to the type of nodule produced and the organisms contained in the nodule.   He believed them all to belong to a single species and applied the name, *Schinzia leguminosarum*. Prior to this, 1877, Frank had stated that the fungus of the root nodules was *Schinzia cellulicola*.

Schroeter, in 1886, was the first to make a division of these organisms into two species.   He transferred them from the genus *Schinzia* into a new genus *Phytomyxa*, and recognized two species: *Phytomyxa leguminosarum* (Frank) Schroeter and *Phytomyxa lupini* Schroeter.   He said that the first species forms nodules on most leguminous plants, *e.g., Trifolium repens, Lotus corniculatus, Orobus vernus*, etc.; while the second forms nodules on *Lupinus luteus* and *L. angustifolius*.

Beijerinck, in 1890, reported that the differences between the organisms of the root nodules were greater than he had earlier supposed, and that *Bacillus Ornithopi*, from *Serradella*, should be recognized as distinctly different from *Bacillus Fabae*, from *Vicia faba*.   This is apparently his first use of the species designation *Bacillus Fabae*.   In earlier papers and also in the same article he referred to this organism as *Bacillus radicicola* var. *Fabae*.

In 1888, he had recognized that not all the organisms are identical, and had listed seven varieties of *Bacillus radicicola*, namely varieties *Fabae, Vicia hirsutae, Trifoliorum, Pisi, Lathyri, Lupini,* and *Cytisi,* as well as mentioning *Phaseolus* and *Robinia.*

Schneider in 1892, described and named five species, with two varieties of one: *Rhizobium mutabile, Rh. curvum, Rh. Frankii,* varieties *majus* and *minus, Rh. dubium,* and *Rh. nodosum.*

Gonnermann, in 1894, secured cultures of several organisms from nodules of leguminous plants which were classified into two species with several varieties of each. Discarding all the earlier nomenclature, he named his organisms *Bacillus tuberigenus* and *Micrococcus tuberigenus.*

Kirchner, in 1895, studied the organism from soybean nodules and concluded that this organism is distinct from the others. The name *Rhizobacterium japonicum* was applied to it.

Many other cases might be cited in which the early scientific workers recognized that not all the nodule organisms of leguminous plants should be classed as belonging to a single species.

At the present time most workers agree that at least two species should be recognized, and a considerable number believe that sufficient differences exist to make necessary the creation of several species. As examples of the second group, recent papers by Dangeard, 1926, and Müller and Stapp, 1925, may be mentioned. Müller and Stapp, on the basis of the morphological, cultural, and physiological characters of the organisms, have separated eleven subgroups of the nodule bacteria of the Leguminosae. No scientific names have been used, nor species designations made. The authors hold, however, that it is possible definitely to identify each subgroup, and suggest that they should be classified on this basis. Dangeard, basing his work largely on careful studies of the nodules, decided that at least 10 species should be recognized. These are given names as follows: *Rhizobium Trifolii, Rh. polymorphum, Rh. Fabae, Rh. Meliloti, Rh. Loti, Rh. simplex, Rh. torulosum, Rh. Phaseoli, Rh. minimum, Rh. Sojae.*

Various workers have formed their classifications on the basis of either one, or a combination of more than one of the following

characters: (*a*) morphology; either on culture media or in the nodule; (*b*) cultural characters; type of colony and growth upon laboratory media; (*c*) physiology; as exhibited in the phenomena of plant inoculation, in the laboratory upon culture media, and in serological reactions.   These characters are generally recognized at present as legitimate bases for species-differentiation.

Robson (1928), in his book entitled "The Species Problem" has brought out some worth-while suggestions regarding speciation.   He considers that species in bacteria are probably of the same order as in higher animals.   But they may well be less permanent from a human point of view and that with the admission of criteria other than morphological, pathogenicity becomes a more valid ground for specific differentiation.

Perhaps the most nearly constant of the physiological properties of the root-nodule bacteria is their ability to cause the formation of nodules upon certain species of the Leguminosae and not upon others.   Certainly, from the practical standpoint, this is one of the most important characters of the organism.   For more than twenty-five years, a utilitarian classification into "cross-inoculation" groups has been in use by students in this field.   In the opinion of the writers, this characteristic is the most logical and valuable one on which to base any differentiation of the genus *Rhizobium* into species.   The character which is now universally used to separate the members of this genus from other bacteria is the ability to form nodules on the roots of leguminous plants.   If a further classification is to be made of the members of this genus, should not this character be used as a species identification?   Detailed studies have shown, also, that the members of each "cross-inoculation" group differ from those of each of the others, in some or all of the following points: morphology, cultural characters, physiological properties, and serological reactions.

It may well be questioned whether the differences which are recognized are of a magnitude to justify specific or only varietal distinctions.   To date no one in the field of bacteriology has been able to define clearly the characters upon which specific separa-

tions are to be based. As in all other fields of natural science, the judgment of the student, based upon, and in conformity with, similar cases, must be the deciding factor. In general, bacteriologists have agreed that a clear and definite difference in some essential character is a just and reasonable basis for species-separation. It is considered that the differences exhibited between the "cross-inoculation groups" are of that magnitude. Also further confusion must arise if the "cross-inoculation group" is to be recognized as a variety instead of a species, for it is clearly recognized that the members of the different "cross-inoculation groups" are further differentiated into subgroups based on cultural, physiological, and serological characters.

In view, therefore, of the fact that the root-nodule organisms naturally fall into several groups, each of which presents definite and constant characters differentiating it from all other organisms, and because of the confusion arising from the present system of classification, it is proposed that several separate species be established.

In the present proposal species-identification is based upon the morphology, cultural characters, physiological properties, and serological reactions of the bacteria. Many detailed reports have been published covering these characteristics, and only a few of the more outstanding points will be mentioned at this time. For more complete descriptions reference is made to the papers of Burrill and Hansen, 1917, Löhnis and Hansen, 1921, Wright, 1925, Stevens, 1925, Müller and Stapp, 1925, and Baldwin and Fred, 1927. Many others might be mentioned. In each case in which sufficient study has been made to render it possible, a species is established for each "cross-inoculation group." In deciding upon the nomenclature appropriate for these species, the authors have been guided by the rules of the International Code of Botanical Nomenclature.

1. *Rhizobium leguminosarum* Frank. The organism causing the formation of nodules upon the roots of *Lathyrus*, *Pisum*, *Vicia* and *Lens*.

a. Growth on mannitol agar is rapid with tendency to spread. Streak is raised, glistening, semi-translucent, and white. Con-

sistency is slimy and occasionally viscous. Considerable gum is formed.

b. Fermentation of carbohydrates. Slight acid-production from glucose, galactose, mannose, lactose and maltose.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules are commonly irregular, with many X and Y shaped forms. Vacuolated forms predominate.

The species designation *"leguminosarum"* was proposed by Frank in 1879, when he erroneously placed the nodule forming organisms in the genus *Schinzia*. The greater portion of his work, as judged by his figures, was done with *Lathyrus* and *Orobus*, and *Orobus* is now placed in the genus *Lathyrus*. Frank recognized only the one species in the genus *Rhizobium*. If new species are to be established, the old species designation *leguminosarum* must be retained, and it would seem appropriate to apply it to the species including the organism causing the root nodules on *Lathyrus*.

2. *Rhizobium trifolii* Dangeard. The organisms causing the formation of nodules upon the roots of *Trifolium* sp.

a. Growth on mannitol agar is rapid. The colonies are white, becoming turbid as they grow older. Often the cultures become so mucilaginous that long threads may be drawn when the growth is touched with a needle. Streak cultures at first show a transparent growth along the line of inoculation. Later this growth becomes mucilaginous and flows down the inclined surface of the agar accumulating as a slimy mass at the bottom. Produces large amounts of gum.

b. Fermentation of carbohydrates. Slight acid—production from glucose, galactose, mannose, lactose and maltose. Usually slightly greater than with *Rh. leguminosarum*.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules are pear-shaped, swollen, and vacuolated. Rarely X and Y shaped forms.

Beijerinck, in 1888, proposed *trifoliorum* as a varietal name for the organism isolated from *Trifolium*. Dangeard, in 1926, used the species term *trifolii*.

3. *Rhizobium phaseoli* Dangeard. Causes the formation of nodules on *Phaseolus vulgaris*, *Ph. angustifolia*, and *Ph. multiflorus*.

a. Growth on mannitol agar is rapid with tendency to spread. Streak is raised, glistening, semi-translucent, white. Consistency slimy and occasionally sticky but not so marked as in *Rh. trifolii*.

b. Fermentation of carbohydrates. Very slight acid-fermentation of glucose, galactose, mannose, sucrose and lactose.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules are usually rods, with few branched forms. They are usually smaller than in *Rh. leguminosarum* and *Rh. trifolii*, and often vacuolated.

Beijerinck, in 1888, was able to distinguish the organisms of Phaseolus nodules from others and applied the term *Bacillus radicicola, phaseolus*-type. He did not, however, give it a definite varietal name. Schneider, in 1892, was the next to propose a specific designation for this organism. He proposed the term *Rh. Frankii* var. *majus* for the organisms symbiotic with *Phaseolus vulgaris*. *Rh. Frankii* var. *minus* was proposed for those symbiotic with *Pisum sativum*. Schneider's terminology is invalid from two standpoints. He failed to utilize any of the earlier specific and varietal names. His characterizations of the organisms are so meagre and conflicting that it is impossible to know definitely the organisms with which he worked. Dangeard, in 1926, used the term *Rh. phaseoli*.

4. *Rh. meliloti* Dangeard. The organism causing the formation of root nodules upon *Melilotus*, *Medicago*, and *Trigonella*.

a. Growth on mannitol agar is fairly rapid but not as fast as that of *Rh. leguminosarum*, *Rh. trifolii*, and *Rh. phaseoli*. Growth is moderate to abundant. The streak is raised, glistening, opaque, and pearly white. Consistency is buttery with considerable gum but usually not viscous.

b. Fermentation of carbohydrates. Strong acid-production from glucose, galactose, mannose and sucrose.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules include both club-shaped and branched forms.

Dangeard first proposed this name in 1926, as applied to the organism symbiotic with members of the alfalfa-sweet clover

cross-inoculation group. Schneider in 1892 proposed the name *Rh. mutabile* for the organisms symbiotic with *Trifolium pratense, T. repens, Melilotus alba,* and *Lathyrus odoratus.* Schneider's terminology is invalid because of his failure to observe the rules of priority, and because of meagre and conflicting descriptions, rendering identification uncertain.

5. *Rh. japonicum* (Kirchner) comb. nov. The organism causing the formation of root nodules on *Soja max.*

a. Growth on mannitol agar is slow and scant under ordinary conditions. Streak is slightly raised, glistening, opaque, and white. Consistency is buttery with little gum formation. Pentose sugars give better growth than the hexoses.

b. Fermentation of carbohydrates. Little if any acid-formation. After prolonged incubation acid from xylose and arabinose.

c. Morphology. Monotrichous flagellation. Bacteroids of nodules are long, slender rods with only occasional branched and swollen forms.

This specific designation for the organism of the soybean root nodules was first proposed by Kirchner in 1895.

The five species described above comprise the list which is proposed at the present time. Specific designations for the organisms from the nodules of other leguminous plants are not proposed now, because of insufficient study and lack of definite information. As the study of these organisms continues, it will undoubtedly be desirable to increase the list of species.

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PROTEOLYSIS AND THE SELECTIVE DESTRUCTION OF AMINO ACIDS BY CLOSTRIDIUM SPOROGENES AND CLOSTRIDIUM HISTOLYTICUM

M. W. MEAD, Jr., AND C. G. KING

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania

INTRODUCTION

The immediate aim of this investigation was to find whether or not a selective destruction of amino acids could be demonstrated in the course of the utilization of natural protein by two typical proteolytic anaerobes. The problem is important because of its relation to the general study of bacterial metabolism and physiology. It has, moreover, some bearing upon the question of the chemical structure of proteins.

There is considerable evidence at hand to show that micro-organisms destroy some amino acids in preference to others when these acids are added separately (in the uncombined form) to culture media (Waksman and Lomanitz, 1925; Jodidi, 1912; Wagner, Dozier and Meyer, 1924; Parsons and Sturges, 1927; Braun and Cahn-Bronner, 1921; Blanchetiere, 1920). A quantitative study of the destruction of tyrosine in proteins has been made by King and Hjort (1926). Since the protein molecule is the normal starting point for the nutrition of proteolytic anaerobes, this appeared to be a more fundamental method of attacking the problem of selective or specific amino acid destruction than to use protein-free media and supply the purified individual amino acids. This method of study is more difficult, however, in that it necessitates the adaptation of analytical methods for quantitative estimation of the amino acids.

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The plan of procedure followed in this investigation involved, first, the preparation of media containing an adequate amount of suitable protein; secondly, it required the determination of the amount of amino acids present (in combination) in each of these media; and finally, it involved another determination of the same amino acids after the growth of an organism had run its course in the media. Differences found in the two series of analyses should give an index to the relative destruction of amino acids for the different acids determined, the different organisms grown, and the different proteins used.

The two organisms, *Cl. histolyticum* and *Cl. sporogenes*, were selected partly on the basis of biochemical and growth characteristics given by Reddish and Rettger (1924). These organisms grow rapidly on protein media and exert a strong proteolytic action. Cultures were obtained from "The American Type Culture Collection" of the John McCormick Institute for Infectious Diseases, Chicago, Illinois. The proteins used were fibrin and edestin, selected as typical animal and plant proteins respectively.

In selecting amino acids for determination, the choice was limited to those few acids for which fairly reliable analytical methods existed by which they could be estimated in protein hydrolyzates. At best there is a wide discrepancy in the literature regarding the quantitative presence in proteins of even such acids as tyrosine, cysteine, tryptophane, and histidine.

Media were prepared by weighing the dry protein into large test tubes. Four grams was adopted as a standard weight of protein to be used in each culture tube. This gave a sufficient quantity of the constituent amino acids for ready determination, and an amount of material which was not too large for ready manipulation. To each tube was then added 35 cc. of a solution of beef extract which was of such strength as to furnish about 0.12 gram of the extract to each tube. The beef extract furnished enough nutrient material to initiate a vigorous growth of the organism and thus insure attack upon the protein. Anaerobic conditions were maintained by placing a petroleum-paraffin seal

on the liquid surface. The tubes were plugged and sterilized either at fifteen pounds pressure, or at ordinary pressure on three successive days.

ANALYTICAL METHODS

In the adoption of analytical methods it became a chief concern to avoid, in so far as possible, interferences by the many unknown metabolic decomposition products which were apt to be present in the mixture to be tested. Hanke and Koessler (1925) described a method by which tyrosine and histidine may be precipitated from protein hydrolyzates—the former as a mercuric chloride complex, and the latter in combination with silver. These acids could in this way be separated from the complex mixture and could be separately estimated by colorimetric methods. For histidine the colorimetric methods of Hanke and Koessler (1919) involving reaction with p-diazo benzene sulfonic acid, was found satisfactory for comparative work. Tyrosine, after separation, was estimated by the method of Folin and Looney (1922). Tryptophane was also removed by a precipitation with mercury and in turn estimated by means of the phenol reagent of Folin and Looney.

Tyramine and histamine, metabolic products derived from the two corresponding amino acids, would be likely to occur in protein culture media. Since these amines are precipitated by mercury and silver respectively, under the same conditions used for the precipitation of the acids, it became necessary to carry out procedures for the separation of each acid and its amine. Such proceedures have been described by Hanke and Koessler (1919 and 1922). They involved the extraction of the amine from an alkaline solution by means of successive small portions of amyl alcohol.

A continuous analytical procedure was developed in the course of which each of the above-mentioned methods was employed. The protein material was first converted to amino acids by acid hydrolysis. The three amino acids were precipitated from aliquot portions of the hydrolyzate. Amyl alcohol extractions were made on solutions of the acids after their separation. The

quantities of acid present were finally estimated colorimetrically, using aliquot portions of exactly 100 cc. solutions. The amount of ammonia present in each culture tube was determined by means of distillation from an alkaline solution through which a small stream of air was simultaneously passed.

It was of considerable importance in the interpretation of results that there should be certainty as to the development in each tube of only the single organism in question. Direct morphological examinations and sugar fermentation tests were made on fresh sub-cultures from the media tubes just previous to analysis.

RESULTS OF AMINO ACID DETERMINATIONS

Percentages of three amino acids found present in several proteins are given in table 1, which summarizes the results of analyses made upon steril controls. Casein has been included for the purpose of checking values obtained here with those quoted by other investigators. In the case of histidine and tyrosine a substantial agreement was found between values obtained here for the three proteins and those previously reported in the literature by the same methods.

Four separate culture tubes were analyzed in each case where a single organism was grown upon a single protein. With one exception, table 2 gives averages obtained after the analysis of four separate culture media. In the case of the growth of *Cl. histolyticum* upon edestin the averages include three separate media only. The figures given represent the percentage of each acid, calculated upon original weight of protein, which had been destroyed. Tyrosine destruction was uniformly in excess of histidine destruction, as shown by the ratios in the middle section of table 2. The third section of the table illustrates the difference in the destructive action of the two organisms upon histidine and tyrosine. It also shows a decided contrast between fibrin and edestin in respect to the ratios characteristic of the two organisms when grown on these two different media. Tryptophane is partially destroyed in the course of acid hydrolysis. Its destruction proved to be so irregular during the hydrolytic process that

no safe conclusions could be drawn with respect to its destruction by the organisms.

DETERMINATION OF SORËNSEN AND AMMONIA VALUES DURING ACTIVE GROWTH

The low values obtained for the destruction of tyrosine and histidine by *Cl. histolyticum* compared to *Cl. sporogenes* indicated that possibly the entire level of amino acid destruction was lower in the case of *Cl. histolyticum* than in that of *Cl. sporogenes*.

TABLE 1

Composition of the proteins used

	CASEIN NO. 1	CASEIN NO. 2	ADOPTED FOR COMPUTATION
Histidine..............................	2.37	2.37	2.4
Tyrosine..............................	4.70	5.43	5.4
Tryptophane...........................	1.03	1.08	1.1
	EDESTIN NO. 1	EDESTIN NO. 2	ADOPTED FOR COMPUTATION
Histidine..............................	2.25	2.23	2.2
Tyrosine..............................	3.95	4.11	4.1
Tryptophane...........................	1.19	1.11	1.2
	FIBRIN NO. 1	FIBRIN NO. 2	ADOPTED FOR COMPUTATION
Histidine..............................	1.82	1.91	1.9
Tyrosine..............................	6.24	6.19	6.2
Tryptophane...........................	2.60	1.80	2.6

Cl. histolyticum, in other words, might be a very strongly proteolytic organism having exceptional ability to hydrolyze protein, but only capable in small measure of deaminizing and otherwise breaking down individual amino acids. If it were found that the putrefactive or decomposition process did not differ markedly in degree for each of the two organisms, then the low destruction of the two amino acids studied would be evidence of a selective action. In order to find to what extent the biochemical processes of the two organisms differed, a series of runs were made in which amino nitrogen and ammonia nitrogen were determined at inter-

vals during the period of most active growth. It was thought that any marked difference in the chemical processes characteristic of these two organisms would of itself be of considerable interest. It was further believed that such work would aid in explaining

TABLE 2

Destruction of amino acids

Average destruction of histidine and tyrosine

CL. SPOROGENES		CL. HISTOLYTICUM	
Fibrin:		Fibrin	
Histidine.....................	23.6	Histidine.....................	22.8
Tyrosine.....................	62.9	Tyrosine.....................	28.6
Av. NH$_3$-N...................	236	Av. NH$_3$-N...................	235
Edestin:		Edestin:	
Histidine.....................	28.4	Histidine.....................	4.5
Tyrosine.....................	52.4	Tyrosine.....................	13.0
Av. NH$_3$-N...................	287	Av. NH$_3$-N...................	248

Ratios of Tyrosine Destruction to Histidine Destruction $\dfrac{\text{Tyrosine}}{\text{Histidine}}$

Cl. sporogenes on fibrin... 2.6
Cl. histolyticum on fibrin.. 1.2
Cl. sporogenes on edestin.. 1.8
Cl. histolyticum on edestin... 2.9

Ratios of Destruction between the Two Organisms

FIBRIN		EDESTIN	
Histidine $\dfrac{Cl.\ sporogenes}{Cl.\ histolyticum}$ 1.03		Histidine $\dfrac{Cl.\ sporogenes}{Cl.\ histolyticum}$ 6.3	
Tyrosine $\dfrac{Cl.\ sporogenes}{Cl.\ histolyticum}$ 2.2		Tyrosine $\dfrac{Cl.\ sporogenes}{Cl.\ histolyticum}$ 4.0	

the cause of a profuse production of tyrosine crystals which almost invariably occurs when *Cl. histolyticum* is grown in protein media.

The results of this work are given in table 3. It will be noted that the runs were made during the first eight days and also at the end of a period of time corresponding to that which elapsed before the amino acid analyses were made.

The table indicates a rather striking difference in amino nitrogen production between the two organisms. *Cl. sporogenes* appears to destroy the excess amino nitrogen as fast as it is formed. *Cl. histolyticum* is somewhat inferior to *Cl. sporogenes* in final ammonia production but the ratio does not approach that of the differences in amino acid destruction. Ammonia traps attached to these culture tubes showed that any ammonia which might have escaped from the tubes during the eight days of active growth in the incubator was negligible in amount.

TABLE 3

Production of amino and ammonia nitrogen

(Milligrams increase over control tubes)

NUMBER OF DAYS AFTER INOCULATION	FIBRIN				EDESTIN			
	Cl. sporogenes		*Cl. histolyticum*		*Cl. sporogenes*		*Cl. histolyticum*	
	NH₂-N	NH₃-N	NH₂-N	NH₃-N	NH₂-N	NH₃-N	NH₂-N	NH₃-N
3	2.0	61.5	34.0	104.0	0.0	15.5	1.1	99.9
5	0.0	72.2	52.4	130.0	0.0	42.4	44.7	123.8
8	0.0	102.6	61.5	127.5	0.0	64.7	58.8	189.1
30 (Visible growth stopped)	27.0	211.1	53.8	154.8	0.0	254.8	85.0	202.1

Final pH values

Table 4 gives the pH values found in certain of the culture tubes after complete growth of the organisms. These measurements were made by the electrometric method with the aid of a hydrogen electrode tube especially designed for small volumes of liquid (Garrison, Henry and Pasternack, 1925).

THE DETERMINATION OF TYRAMINE AND HISTAMINE

It was found that tyramine could be estimated by the use of the phenol reagent. A special quantitative study has been made of the production of this amine in certain protein media. In table 5 a parallel comparison is made of total tyrosine destruction and the amount of this tyrosine appearing in the medium as tyramine. Percentages are based on original weight of protein.

It is to be noted that in the first example among the figures given for beef there is an approximate equivalence between tyrosine destroyed and that appearing as tyramine. This is true also for the casein determinations in the *Cl. sporogenes* column. When the total tyrosine destruction becomes greater,

TABLE 4

pH values of media after growth

MEDIUM	pH	E.M.F.
Edestin—*Cl. sporogenes*, No. 3	8.2	0.822
Edestin—*Cl. sporogenes*, No. 4	7.9	0.802
Fibrin—*Cl. sporogenes*, No. 3	7.8	0.798
Fibrin—*Cl. sporogenes*, No. 4	7.5	0.778
Edestin—*Cl. histolyticum*, No. 3	7.6	0.788
Edestin—*Cl. histolyticum*, No. 4	7.6	0.788
Fibrin—*Cl. histolyticum*, No. 3	7.6	0.782
Fibrin—*Cl. histolyticum*, No. 4	7.6	0.784

TABLE 5

Relation of tyramine found to tyrosine destroyed

MEDIA	CL. SPOROGENES		CL. HISTOLYTICUM	
	Per cent tyrosine destroyed	Per cent tyrosine found as tyramine	Per cent tyrosine destroyed	Per cent tyrosine found as tyramine
Beef	34.5	31.0	58.6	27.6
	58.6	27.6		
	58.6	31.0		
Casein	31.5	33.3	55.5	24.1
	35.2	29.6		
Edestin	58.5	34.3		
	63.4	31.9		

the amount of tyramine present does not increase correspondingly. This would indicate that decarboxylation or tyramine production was a first step or stage in the degradation of tyrosine by the two organisms and that tyramine was then further consumed in the putrefactive process. Histamine was not found present in any of the cultures examined.

DISCUSSION OF RESULTS

In considering the problem of selective destruction of amino acids by the two organisms, it is evident that there is a distinct difference between *Cl. sporogenes* and *Cl. histolyticum*. Differences of this kind might be interpreted as the result of a divergence between the two organisms in their degree of development upon the media were it not for the amino and ammonia nitrogen data obtained. The latter indicate clearly that the degree of growth or metabolism was comparable for both organisms, providing that ammonia production may be taken as an approximate index to the destruction of amino acids. The difference in destruction between the two organisms is particularly striking for the edestin media. The differences in amino acid destruction are far greater than the differences in ammonia production.

There is also a marked difference evident in the destruction of histidine and tyrosine contained in the two proteins, fibrin and edestin. The ammonia production is only slightly greater for edestin than for fibrin, yet the destruction of the two amino acids studied is distinctly lower for *Cl. histolyticum*. For *Cl. sporogenes* the ammonia difference is greater but the difference in destruction, of amino acids between the two proteins, is negligible. It is evident then that the differences in the selective destruction of amino acids by organisms may be dependent upon, or closely related to, the nature of the protein furnished by the media.

Amino nitrogen values have shown *Cl. histolyticum* to be more actively proteolyitc than *Cl. sporogenes*. Such a tendency toward the production of free amino nitrogen is doubtless significant in relation to the phenomenon of tyrosine crystallization. An increase in free, uncombined tyrosine would ordinarily be expected to accompany an extended breaking up of the protein molecule. Sano (1926) has furthermore found that tyrosine possesses a low solubility in the pH range shown to exist in the media of *Cl. histolyticum*. Any appreciable amount of tyrosine set free during the growth process would be expected, if not otherwise utilized, to crystallize from solution. In the case of the two media studied there was a fairly high amino nitrogen production by *Cl. histoly-*

ticum compared to that by *Cl. sporogenes*, which, for the most part was zero. Furthermore tyrosine destruction in the *Cl. histolyticum* cultures was unmistakably lower than in cultures of *Cl. sporogenes*. Considering the solubility of tyrosin in relation to known pH, these facts make the appearance of tyrosine seem not in the least surprising. It is possible, however, that tyrosine is preferentially liberated by tyrosine-crystal forming organisms in their disintegration of the protein molecule.

CONCLUSIONS

1. *Clostridium sporogenes* and *Clostridium histolyticum*, when growing in protein media, tend to utilize or destroy the amino acid tyrosine in greater degree than they utilize or destroy histidine.

2. *Clostridium histolyticum* destroys neither of these two amino acids to the same extent that *Clostridium sporogenes* does. The action of the organism is selective toward these two particular acids in fibrin and edestin media.

3. The degree of destruction of tyrosine and histidine by *Clostridium histolyticum* varies with the protein used.

4. The proteolytic action of *Clostridium histolyticum* is greater than that of *Clostridium sporogenes*. The former, however, is not greatly inferior to the latter in putrefactive power as indicated by the production of ammonia.

5. The two organisms give rise to a hydrogen ion concentration in protein-rich media ranging between pH values of 7.5 and 8.2. In *Clostridium histolyticum* cultures, the hydrogen ion concentration is such as to facilitate tyrosine crystallization, whenever this acid is set free in the medium.

6. Tyramine is one of the products of tyrosine utilization by these organisms. It probably is further decomposed to a limited extent after arising as a primary decomposition product of tyrosine.

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THE PROBLEM OF DILUTION IN COLORIMETRIC H-ION MEASUREMENTS

I. ISOHYDRIC INDICATOR METHODS FOR ACCURATE DETERMINATION OF pH IN VERY DILUTE SOLUTIONS

EDNA H. FAWCETT

Junior Pathologist, Bureau of Plant Industry, United States Department of Agriculture

AND

S. F. ACREE

Principal Scientist (Chemist), Agricultural Waste Products, Bureau of Standards

INTRODUCTION

Both chemical and biological literature show increasingly the need for more exact and dependable methods of colorimetric procedure for H-ion measurements (checked by the E.M.F. method when possible) on water and on very dilute solutions beyond the usual range of 0.2 to 0.02 M.

This was recognized by Birge and Acree in 1918 when they emphasized the necessity for adjusting the pH of the indicator for use with weakly buffered solutions, and of employing what we shall henceforth call the isohydric indicator method (abbreviated to iso-indicator or i. i. method) to obtain the final correct pH readings for standards and unknowns. This result is accomplished by the use of adjusted indicator solutions having nearly or exactly the same pH that the solution or water under examination shows from preliminary tests. In a study of the pH of water and solutions used in intravenous medication Williams and Swett in coöperation with Mellon, Slagle and Acree in 1920–1921 prepared excellent double distilled water showing pH values of about 6.8 to 7.1 when tested with brom thymol blue adjusted to about pH 7.0. This water gradually absorbed carbon dioxide from the air and generally reached a pH of about 5.7 to 6.0.

Brightman, Meacham and Acree in 1919–1920 combined E.M.F. and spectrophotometric methods to correct the data for the salt errors of indicators in such work.

That bacteriologists are beginning to feel the same need is shown by the statement made in the last Manual of Methods issued by the Society of American Bacteriologists (p. c. 45, Committee on Technic, 1927). In the discussion of the colorimetric procedure the committee points out that "if insufficient buffer is present or if either the distilled water or the indicator solution should be too far from the desired reaction, the result may be misleading." We also see occasional references by biological workers, who are using colorimetric methods, to the pH of the water used and to the means by which the water was prepared.

The list of problems to which the kind of technique developed in the present investigations is applicable is large and includes pH tests on plant extracts, weakly buffered culture media like corn meal agar (especially when it is necessary to dilute the latter more highly than usual to eliminate color or turbidity), tests on dilute buffer solutions, natural and purified drinking water, boiler water, sewage, factory waste liquors, solutions of sugar during the refining process, distilled water used in industries including the manufacture of beverages, and solutions for ore flotations.

In such problems workers often fail to recognize that the colorimetric technic heretofore employed is inadequate for determining the pH of water or of very dilute solutions. The data presented in this article show the latter to be true for the following several important and fundamental reasons.

1. The "salt errors" or "buffer corrections" that must be applied to the buffer-color standards and the unknown solutions of 1.0 to 0.02 M are of an entirely different order from those occurring in solutions of less than 0.001 M.

2. The adjustment of the pH value of the indicator solution itself is without practical influence on the standard buffers and unknowns to which it is added in making colorimetric tests on the usual solutions of 0.2 to 0.02 M. Such adjustment is strikingly

necessary, however, because of its marked effect, with solutions which are so dilute that the added indicator produces considerable change in the total buffer and H-ion content. A method of adjusting the indicators was therefore devised.

3. In the usual colorimetric work the effect of the carbon-dioxide and other impurities in the distilled water used for making the solutions or dilutions thereof may be neglected, but in very dilute solutions these factors are of marked influence. As shown in these investigations, widely different effects may be produced by variations in the character or condition of the water used; for example, water containing considerable CO_2 as when freshly collected from ordinary stills (usually about pH 5.0), water which has reached equilibrium with the CO_2 in the air (generally about pH 5.7) or CO_2-free water in the neighborhood of pH 7.0, all of these values being subject to more or less variation due to the presence of small quantities of sprayed-over solids. Methods were devised for preparing experimentally and for testing all these "kinds" of water.

4. In view of the three foregoing phases of the problem it is clear that we are in great need of accurate quantitative colorimetric and E.M.F. data on the dilution of the important buffers, culture media and indicators with these different types of water in order to discover and test more fully the laws underlying the hydrolysis and ionization of buffers and indicators by water. Accordingly, the accompanying tables and curves are here presented for possible assistance to others.

HISTORICAL

This coöperative work was begun in January, 1924, in order to apply the isohydric indicator methods and explain and rectify the source of errors which Miss Fawcett had previously observed in her work (covering about 400 experiments) on the dilution of culture media. She had shown in 1923 that stock indicator solutions standing in soft glass bottles gradually become more alkaline and give readings different from those of either fresh indicator solutions or old ones readjusted with acid to the first faint color change. These differences were very pronounced and

variable in her work on certain solutions with inherently weak buffer properties and on others diluted with (a) ordinary distilled water, (b) reboiled distilled water and (c) double distilled water. Under these conditions it was impossible to determine the correct pH values. Similar results had been obtained by Acree and his coworkers. The isohydric indicator method offered the only correct means of (a) preparing, studying, keeping and using distilled and superpure water; of (b) measuring the actual pH thereof and of buffer solutions diluted therewith; of (c) studying the salt errors of indicators; of (d) making reliable comparisons of colorimetric pH values with the corresponding E.M.F. data; and of (e) studying solutions involving the carbon dioxide-bicarbonate-carbonate ($CO_2 - NaHCO_3 - Na_2CO_3$) equilibrium, especially when neutral gases are passed therethrough and into pure water to investigate the CO_2 equilibrium. This article is the first of a series of our researches on the above subjects.

Important work having a bearing on this general subject has been done by other investigators, notably Kolthoff (1925), who studied the effect of the dilution of buffers on indicator salt errors; McBain, Dubois and Hay (1926) in their paper on the "Salt Error[1] of Indicators Caused by Standard Alkaline Buffers Themselves," and the earlier work of McClendon (1917) and that of Wells (1920) on sea water. A paper dealing specifically with the adjustment of indicators for use with weakly buffered solutions has recently appeared by Pierre and Fudge (1928), who worked out both colorimetric and electrometric methods of adjusting the phenolsulphonphthaleins to their mid-points when determining the pH of soil extracts. Schlegel and Steuber (1927) recognized the effect both of CO_2 and of the reaction of the indicator on their determinations of weakly buffered sugar solutions, and attempted to adjust their brom thymol blue indicator to pH 7.0. Karraker (1923) and Marsh (1924) also published notes suggesting the adjustment of indicators to their mid-points.

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[1] In certain of their experiments the so-called salt errors were actual pH changes caused by the neutralization of their alkali standards by the free indicator acids used.

Their work grew out of a need for greater accuracy in colorimetric work with soil extracts.

Because of the impracticability of publishing in one article, (*a*) the many parallel determinations which have been necessary to study fully the effect of CO_2 and of the indicator acids on the pH of dilute solutions from a colorimetric standpoint, and (*b*) the complete electrometric data necessary for calculating the *salt errors*, we have reserved this phase of the subject for another paper in which the relation of our work to all previous work on salt effects will be discussed.   This paper is now nearly completed and will clarify some points left in doubt by the present paper, such as the accuracy of indicator adjustments, and of pH values assigned to water containing small amounts of impurities.

TECHNIC

Since the accuracy of colorimetric work with dilute or weakly buffered solutions depends entirely on the use of the proper technic, the methods found best suited to the needs of the experiments described in this paper must be given in detail at the risk of tedium.

1. Glassware

All glassware used was Pyrex, with the exception of some of the graduated pipettes.   The *flasks* were prepared as follows: They were heated in the Arnold Sterilizer for about 1 hour successively with (1) $N/100$ alkali, (2) $N/100$ acid and (3) distilled water, rinsing carefully between (2) and (3).   They were then emptied and drained while hot and plugged with cotton to keep out dust.

Tubes after having been cleaned by a thorough laboratory routine, were given an extra treatment as follows: They were placed in large pyrex beakers, filled and covered with distilled water which was drained off, refilled and covered with fresh distilled water, steamed for one hour in the Arnold Sterilizer, emptied and drained on removing from the steamer, dumped on a clean towel and placed quickly while very hot in clean wire baskets in an inverted position.   In this way the tubes dried almost immediately without retaining much of the rinse water.

Pipettes were carefully selected, cleaned, rinsed repeatedly with distilled water and tested with an indicator to determine whether the glass changed the color of the indicator. If a small quantity of brom cresol purple adjusted to pH 5.7 (which is the pH of water at equilibrium with the CO_2 in the air) did not become bluer on standing for 5 or 10 minutes in the pipette it was considered safe for use. We recommend the use of Pyrex pipettes and burettes after our very satisfactory work with a few of them.

2. Water

Super-pure water[2] for use in the dilution experiments was made with a pH of 6.9 to 7.1 in a special commercial still and was also prepared from water from this high capacity still by redistillation in a specially designed table or desk still. The latter, with the exception of the distilling flask and condenser jacket (which were of glass), was constructed entirely of block tin. The water was distilled from a pyrex flask through tin rings in a tin column, cooled in a tin condenser, and collected in pyrex flasks. No chemicals were added to the water in the distilling flask but aeration by means of air led through a special type of soda lime jar arranged to remove all CO_2 and soda lime dust was carried on continuously both in the distilling flask and in the collecting flask during the distillation process. In this way water, entirely free or nearly free from CO_2, could be collected and tested immediately on removal of the collecting flask. Both of these stills and the soda lime jar will be described in a separate paper.

The *water was always tested* by the technic of section 5 (*d*) with a solution of brom thymol blue adjusted to pH 7.0 and the readings varied between pH 7.0 and pH 7.05 with the exception of the first portions coming over, which were sometimes more alkaline, or more acid, due to incomplete aeration as proved by further aeration. These slight variations in pH were apparently not due to temperature, since no appreciable change could be detected experimentally by means of the indicator over a range of 17° to 25°C.

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[2] *Note:* Conductivity experiments to be published in another article show this water to be superpure.

The earlier prevalent belief (for example, Dawson, 1925) that the customary distillation of about one-third of ordinary distilled water will drive out the carbon dioxide and leave a nearly neutral or buffer-free pH 7.0 water rests upon the failure to recognize the presence of sprayed-over alkaline impurities still in the residual water.   Consequently, in making pH tests of such water with the usual unadjusted mono sodium brom and thymol blue having a pH value of possibly 5.9 to 6.2, it is not always understood that the added indicator may be partially neutralized by the residual carbonate-bicarbonate buffer salts of possibly pH 7.5 to 8.5 in the water to give a reading which is close to pH 7.0 and is mistaken for that of the water itself.

This influence of both indicator and water on the final pH reading is shown by the fact that superpure pH 7.0 water gave the reading pH 4.6 with brom phenol blue of pH 3.4; registered pH 5.7 with brom cresol purple of pH 5.2; showed a pH 7.05 with brom thymol blue of pH 7.0; and had the pH 8.05 when mixed with phenol red of pH 8.6.   This same change of the pH reading of the indicator is shown in the tables and columns for the highly diluted buffers, which may of course be considered as slightly impure water.   Neutral salts like sodium chloride in such minute concentrations have no appreciable influence on the neutrality or pH of the superpure water and its solutions but are non-buffer impurities that increase the conductivity.   Hence a statement should be made at this place to the effect that the only way the pH of water containing very small quantities of alkaline salts (pH 7.0 to 9.0) or of CO_2 (pH 5.0 to 7.0), or of both, can be determined accurately or even approximately is by adding 0.2 cc. of each member of a graded pH series of phenol red, brom thymol blue or brom and cresol purple solutions to 10 cc. portions of the water and finding the point or zone at which there is agreement between the pH of one of the adjusted indicator solutions and its pH after mixture with the water under test and comparison with the buffer-color standards.

The following method was used for keeping water free from CO_2 during an experiment.   The pyrex flasks were fitted with double perforated thoroughly cleaned rubber stoppers.   In one opening

was inserted a pyrex tube through which CO_2-free air was led into the water by means of a rubber tube connected with the soda lime jar. The air was thus allowed to bubble through the water at a fairly rapid rate and escape through the free opening of the stopper. Water which was allowed to stand without aeration for several hours in these flasks, even when tightly closed with rubber stoppers, reabsorbed CO_2 to some extent through the stoppers (the pH falling from 7.0 to 6.8 or 6.5 in eighteen to forty-eight hours) and had to be reaerated before use if CO_2-free water were desired. In the case of redistilled water which had reabsorbed CO_2 (pH 6.4 to 6.8), aeration at room temperature for one hour was found to be in most cases a safe procedure for removing all traces of CO_2, while even ten or fifteen minutes' aeration would often bring it to within 0.05 pH of its CO_2-free equilibrium point if the rate of bubbling was fairly rapid. Ordinary distilled water on the other hand, which on collection is supercharged with CO_2 and has a pH of about 5.0, can only be freed completely of carbonic acid by thorough boiling (usually about ten minutes per liter) and aerating immediately while hot or by aerating for a much longer time (five or six hours) without heating. By cooling the water in the flask in a flowing cold water bath while the aeration is being carried on, it can be brought to room temperature rather quickly.

The same general method of handling the water was used in *controlling CO_2 equilibria for experimental purposes.*

1. To obtain water in equilibrium with the CO_2 in ordinary fresh air, a flask containing tested redistilled water at room temperature was saturated with the air which had been taken from a pressure cock (after blowing it off) and had been passed through a side neck filter flask filled with absorbent cotton to rid it of condensation moisture or other impurities. When redistilled water treated in this manner was tested by the technic of Section 5 (*d*) with brom cresol purple adjusted to pH 5.7 the equilibrium point was generally reached in about ten minutes and found to be pH 5.7 at temperatures between 25° and 30°C.

2. Since repeated tests showed that water freshly collected from ordinary stills contained more CO_2 than it did after standing in an

open vessel and coming to equilibrium with the CO_2 in the air, and since the distilled water was found to be practically constant at pH 5.0 for the still and tap water used in our laboratory, it was chosen as one of the "kinds" of water to be used experimentally in making dilutions. Accordingly pH 5.0 water was prepared artificially by adding to 200 cc. of tested CO_2-free redistilled water, 1 cc. of the same water which had previously been saturated with CO_2 from a tank containing the compressed gas. An experimentally constructed curve for pure water containing graded quantities of CO_2 will be given in another paper.

3. Adjustment of indicators

The adjusted series of indicators were prepared by the methods first devised and recommended by one of us and his co-workers in their introduction and development of the use of sulphonphthalein indicators (Lubs and Acree, 1916; Acree and Slagle, 1908; White and Acree, 1918). These methods involve, first, the preparation of a stock solution of each of the mono-sodium uncolored indicator salts now adopted generally by other workers; (see Clark, 1920, p. 14) and secondly, the adjustment of several portions of each of these stock solutions to the desired hydrogen-ion values, preferably 0.2 pH apart, a procedure not yet generally adopted but absolutely necessary in accurate colorimetric work with dilute solutions.

The stock solutions of the indicators with two exceptions (methyl red and brom phenol blue) were prepared by grinding them in a mortar, or warming them, with a volume of $N/20$ alkali equivalent to 1 mol of indicator, i.e., sufficient to dissolve them or at most give a slight color change and then diluting with good distilled water to the strength desired, 0.02 or 0.04 per cent, as is the general practice. These uncolored or slightly colored stock solutions served as a starting point for further neutralization with standard alkali and graded adjustment of the pH values and colors. We have found it convenient for accurate colorimetric work with dilute solutions to have constantly on hand sufficient adjusted portions of each stock indicator to include its lowest,

TABLE 1

Adjustment of indicators

Brom phenol blue

pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*
		cc.	cc.
3.4	0.5	1.5	1.5 / 1.5
3.6	0.82	2.46	2.46 / 2.50
(3.7)†	1.00	3.00	3.00
3.8	1.07	3.21	3.00 / 3.10 / 3.21
4.0	1.28	3.84	3.50 / 3.55
4.2	1.45	4.35	3.84
4.4	1.60	4.80	4.07
4.6	1.72	5.16	4.35

Brom cresol green

pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*
		cc.	cc.
(4.2)†‡	1.00	2.80	2.8
4.2	1.23	3.44	3.17
4.4	1.34	3.75	3.44
4.6	1.45	4.06	3.75 / 3.30
4.8	1.58	4.42	4.06 / 3.80
5.0	1.68	4.70	4.20 / 4.05
5.2	1.78	4.98	4.42
5.4	1.84	5.15	4.70 / 4.30
5.6	1.90	5.32	

Brom thymol blue

pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*
(5.9)†	1.00	3.20	3.20
6.2	1.13	3.61	3.45 / 3.61 / 4.10
6.4	1.20	3.84	3.70 / 3.84
6.6	1.29	4.13	3.95 / 4.13 / 4.30

Brom cresol purple

pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*
(5.0)†	1.00	3.70	3.70
5.2	1.07	3.97	3.97 / 3.75
5.4	1.11	4.10	4.10
5.6	1.17	4.33	4.33 / 4.25 / 4.20
5.8	1.24	4.59	4.59

TABLE 1—*Continued*

Brom thymol blue · Brom cresol purple

pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*	pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*
		cc.	cc.			cc.	cc.
6.8	1.39	4.44	4.20 / 4.44 / 4.80	6.0	1.33	4.92	5.00 / 4.92 / 4.90
7.0	1.50	4.80	4.65 / 4.80 / 5.00	6.2	1.45	5.36	5.36 / 5.30 / 5.40
7.2	1.63	5.21	4.95 / 5.21 / 5.15	6.4	1.56	5.77	5.77
7.4	1.73	5.53	5.45 / 5.53	6.6	1.68	6.21	6.21 / 6.0 / 6.0
7.6	1.81	5.79	5.52 / 5.79				

Cresol red · Phenol red

pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*	pH of adjusted indicator	Molecular equivalent of alkali per decigram (calculated from curve)	N/20 NaOH per decigram (calculated from curve)	N/20 NaOH per decigram actually added*
(6.6)†‡	1.00	5.3	5.3	(6.6)†	1.00	5.70	5.70 / 5.70
7.2	1.075	5.69	5.55* / 5.88*	7.0	1.11	6.32	6.57 / 6.70
7.4	1.11	5.88	5.8 / 6.20	7.2	1.16	6.61	7.01 / 7.20
7.6	1.17	6.20	6.30 / 6.62	7.4	1.24	7.07	7.72 / 7.70
7.8	1.25	6.62	7.05	7.6	1.35	7.69	8.21
8.0	1.33	7.05	7.05 / 7.68	7.8	1.44	8.21	8.95
8.2	1.45	7.68	7.55 / 8.27	8.0	1.57	8.95	8.70 / 9.69

TABLE 1—*Concluded*

pH OF ADJUSTED INDICATOR	MOLECULAR EQUIVALENT OF ALKALI PER DECIGRAM (CALCULATED FROM CURVE)	N/20 NaOH PER DECIGRAM (CALCULATED FROM CURVE)	N/20 NaOH PER DECIGRAM ACTUALLY ADDED*	pH OF ADJUSTED INDICATOR	MOLECULAR EQUIVALENT OF ALKALI PER DECIGRAM (CALCULATED FROM CURVE)	N/20 NaOH PER DECIGRAM (CALCULATED FROM CURVE)	N/20 NaOH PER DECIGRAM ACTUALLY ADDED*
Cresol red				Phenol red			
		cc.	cc.			cc.	cc.
8.4	1.56	8.27	8.95	8.2	1.70	9.69	10.09
8.6	1.69	8.95	9.43	8.4	1.77	10.09	9.2–10.43
8.8	1.78	9.43		8.6	1.83	10.43	10.93

pH OF ADJUSTED INDICATOR	MOLECULAR EQUIVALENT OF ALKALI PER DECIGRAM (CALCULATED FROM CURVE)	N/20 NaOH PER DECIGRAM (CALCULATED FROM CURVE)	N/20 NaOH PER DECIGRAM ACTUALLY ADDED*
Methyl red			
4.6	Unadjusted alcoholic solution		
4.8	1.0	7.4	7.4
5.0	1.25	8.18	{ 8.0 / 9.25 }
5.2	1.42	9.27	{ 9.5 / 10.5 }
5.4	1.58	10.33	{ 10.5 / 11.7 }
5.6	1.68	10.98	12.5
5.8	1.82	11.90	13.5

Note: The theory of the proper adjustment of these indicators to be given more fully in a later article will explain the discrepancies in the above tables between actual and calculated amounts of alkali necessary to reach a given pH value.

* Values included in braces represent different adjustments of the same sample or of different samples of the indicator.

† Parenthetical values were determined on 1 mol stock solutions (per se) and do not necessarily correspond to the calculated molecular equivalents.

‡ Determination made on a separate lot of indicator from those represented by the values which follow.

mid-point and highest useful pH values, or still better to cover its entire color range in steps of 0.2 pH.

The *details of the adjustment* were as follows: The stock solution of each indicator was divided into 100 cc. portions and placed in 100 cc. Erlenmeyer flasks to which were added graded amounts of N/20 NaOH calculated from the theoretical ionization constants or titration curves of the indicators to bring the solutions to the desired pH values preferably 0.2 pH apart (table 1). The dilution error is negligible. The fact that the indicator samples were not always free from impurities made it necessary to devise a method of checking the pH values thus obtained.

The following procedure was found to be quite simple and satisfactory. This experimental application of the isohydric indicator method was developed by Miss Fawcett and is much better than the use of drops on white test plates or in shallow pyrex dishes (Brown, 1924) or than the usual comparisons in 16 to 18 mm. test tubes or in a Duboscq colorimeter or by Gillespie's (1920–1921) drop ratio method. (1) A volume of 0.2 cc. of the adjusted indicator to be tested for pH was introduced into the bottom of a tube test. (2) The same volume was placed in a second test tube to which was also added 0.2 cc. (even 0.05 cc. is sufficient) of a M/20 (or even M/1000) buffer having the same desired pH value to form the buffer-color standard. The two test tubes were then stoppered and quickly brought to a horizontal position over white paper or porcelain to cause the solutions to spread out in thin layers of substantially equal area.[3]

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[3] Although the volumes in (1) and (2) are different, according to Beer's law and in actual practice the colors match at the same pH. In the equation $H \times In/HIn = Kind$ expressing the concentration of the colored ions In and molecules HIn and H-ions in terms of the indicator ionization constant Kind, the H is the same for both solutions at the same pH value. Therefore the fraction In/HIn is also constant at this H value, and for equal areas viewed the colors of the two solutions are the same regardless of the difference in the volume and depth. Conversely, if the colors are alike, the pH value of the indicator solution may be considered as accurately adjusted to that of the standard buffer-indicator mixture, which however may not be free from salt errors. Since the hue of the indicator also changes greatly with pH, this isohydric indicator method gives a remarkably easy way of adjusting the pH and colors of these very dilute indicator solutions to those of known reproducible buffer-color standards.

In this way the colors were readily compared. If the indicator solution under test did not match the same solution when mixed with buffer, it was further corrected by adding more alkali or acid, cautiously, to the flask and retesting until the colors were exactly the same. If only part of the series is adjusted theoretically before beginning testing, enough of the stock solution may be reserved for making the corrections and completing the series as desired. The adjustments may be made entirely in an empyrical way with success but it is a help to have the theoretical values from the titration curves or our table 1 as a guide.

As shown in table 1, the stock solutions made with one mol-equivalent of alkali were usually found to be near the acid end of the range. Brom phenol blue and methyl red were exceptions to this rule. In the case of the former it was found that a quantity of alkali equivalent to only 0.5 mol of the indicator was sufficient to bring the solution to pH 3.4 which falls a little short of being the acid end of its range. In the case of methyl red hydrochloride the unadjusted indicator matched the indicator-buffer mixture pH 4.6 and was used as number 1 in that series. Where the amounts of alkali actually added in adjusting the indicators differed from the theoretical quantities they are given in parallel series.

When adjusting the indicators for alkaline ranges, viz., brom thymol blue, phenol red and cresol red, it was necessary to do the matching rapidly unless the test tubes were first filled with CO_2 free air, because the indicator solution containing no buffer absorbed CO_2 readily from the air when spread out in a thin layer. With practice, however, this can be done successfully. We shall later report on the use of graded amounts of sodium bicarbonate in buffers and indicators to keep them at air-CO_2 equilibrium.

Unfortunately some of the solutions were found to be very unstable for reasons not fully understood, especially in the alkaline ranges above pH 7.0. The changes which took place were, in the cases of brom thymol blue, phenol red and cresol red, nearly always in the acid direction but their occasional irregularity seemed to point to some cause or causes other than absorption of

CO_2, though it is possible that a gradual action of the latter in conjunction with uneven distribution of colloidal or particulate impurities from the indicators or accidental introduction of particles of dust, bits of cork or occasional slight fungus growth may be responsible for this instability.   These alkaline solutions required continual readjustment by the addition of more alkali, or of acid in a few cases.   One sample of brom cresol purple always gave solutions which became more alkaline on standing.

In practice the series of indicators being used in any given experiment was checked by the method described on page 175 and readjusted when necessary.   Results did not seem to indicate that the increase in buffer content or the dilution of the solutions thus readjusted was sufficient to affect the readings.   This point was checked with care and indicator solutions to which a double portion of alkali had been added gave identical readings in pure CO_2-free water with those having the same pH values, but containing only the original calculated amounts of NaOH.   Uniformity of readings made on redistilled water under similar conditions with different lots of adjusted and readjusted indicators furnish ample proof of the accuracy of the method.

4. Technic for making dilution series

The following procedure was used in making the dilution series. The pH of the undiluted medium or buffer was always determined electrometrically.   A double hydrogen electrode was used in making all pH determinations together with a M/10 sodium acid phthalate solution (pH 3.95 to 3.97 as determined by different workers) as the standard.   The same standard solution was used in making electrometric determinations throughout the experiments covered in this publication.

Clark and Lub's buffers were used in the colorimetric standards and these were also checked by means of the hydrogen electrode. Accuracy to within 0.03 pH was attempted and care was taken that slight errors in value were all in the same direction in order to keep the intervals even.   The pH 7.0 buffer actually did not vary more than $\pm$ 0.01 pH during the entire period of experimentation.

Buffer-color standards were made up at frequent intervals by adding 0.2 cc. of the indicator to 10 cc. of buffer. The color standards used in any given set of experiments were always made with one, usually the middle one, of the adjusted series of indicators used in the unknown solutions under test. This takes care of the variation in the purity of commercial indicators.

In the method of making dilutions, a large flask of redistilled water (1) was kept aerated with CO_2-free air from the soda lime scrubber at pH 7.0. Two more flasks, (2) and (3), were prepared at pH 5.7 and pH 5.0 respectively from the same redistilled water, as described under *Technic, Sec. 2, Water*. It was found that water containing sufficient CO_2 to register pH 5.0 would not lose its acidity to any appreciable extent during the progress of an experiment if kept in a closed flask. Having prepared these three "kinds" of water, the lower dilutions for each series could be made directly and the higher ones prepared from a 1:10 or 1:100 dilution with water which was kept aerated with (1) CO_2-free air or (2) ordinary air, as the case might be, or (3) simply kept in a closed flask when pH 5.0 water was needed.

5. Procedure for making the colorimetric tests and calculating the pH values

a. A volume of 0.2 cc. of the indicator was introduced from a graduated pipette into the bottom of the tube.

b. The required amount of buffer or medium or dilution of the same or of water was added to the indicator by holding the tip of the pipette always just below the surface while adding the liquid to prevent loss or reabsorption of CO_2. By introducing the indicator first and filling carefully, as described, the color becomes evenly distributed without shaking or inverting the tube and in this way chances for changing the CO_2 equilibrium are lessened. Dilutions and comparisons with the usual buffer-color standards were made singly or in groups of two or three as quickly as possible to minimize changes in CO_2 content. The test tubes may be first filled with CO_2-free air when working in alkaline ranges and with super pure water.

c. A free circulation of fresh air in the room is essential when

carrying on an experiment with CO_2-free water. Reabsorption of CO_2 in the tubes is so rapid under any other condition (as when the air is close and impure or when there are gas flames in the room) that it is often impossible to obtain good readings. The rather rapid action of CO_2 entering the liquid from the atmosphere can be readily detected by the appearance of the "acid" color of the indicator at the top of the liquid in contrast to the color below.

d. The tables show that in studying the mutual effect of the water, buffer and indicator in giving a resultant final hydrogen-ion concentration, pH readings were made on each buffer dilution, and on the water, mixed with each and every adjusted solution of the indicator or indicators falling in the pH range measured. Since all these pH readings differ, we must analyze the data to find the actual pH of the diluted buffer or the water containing no indicator. According to the isohydric principle, buffers and indicators unlike in pH change each other into a resultant interme-diate pH; the correct pH, barring salt errors, is found only when the pH of one of the indicators is the same before and after the addition of the water or solution under test. But to estimate this correct pH point or zone, the unknown solution need not be treated with *all* of the different pH steps of the given indicator. For ordinary practical work it will be found sufficient to have a short series of three solutions of each indicator adjusted at or near the lowest, midpoint and highest useful pH points of its range, say at pH 6.0, 7.0 and 7.6 for brom thymol blue. For more precise work the complete 9-point series with intermediate steps 0.2 pH apart should also be made. The convenience and relative usefulness of the short and the complete series depend greatly on the concentration of the buffer in the unknown solution and can well be illustrated by table 5, giving the tests of the pH values of dilute phosphate buffers with phenol red and cresol red.

The electrometric pH of the м/20 phosphate was adjusted to 7.82. The first colorimetric test of the unknown should always be made with the midpoint indicator solution, or pH 7.8 phenol red in this case, to see whether the solution shows a pH below, at or above the midpoint pH. Table 5 shows that the low, high and

all intermediate pH points of phenol red and cresol red gave the reading pH 7.8+ with the 1:5 and 1:10 dilutions.   We conclude then that it is practically immaterial what the pH adjustment of the indicator is for measuring phosphates more concentrated than M/200.   At a dilution of about 1:100 (M/2000) the pH 7.0 and 8.6 indicators slightly lower or raise the pH of the phosphate and give the readings pH 7.8 and 7.85.   A dilution of 1:1000 (M/20,000) is typical of cases where the short or 3-point or 3-step or 3-pH phenol red series is both necessary and sufficient.   The midpoint or pH 7.8 phenol red gives a resultant reading 7.7, thus showing that the pH 7.82 of the M/20 phosphate buffer has been decreased by the pH 7.0 water on dilution, and is below 7.7.   The lowest or pH 7.0 phenol red gives the reading 7.5, thus proving that the pH of the diluted phosphate buffer is above 7.5.   As the diluted phosphate itself is above pH 7.5 and below pH 7.7, or about three-fourths way from the pH 7.0 to 7.8 indicator, we use three-fourths as a factor on the difference of 0.2 pH between the pH 7.5 and 7.7 readings and choose 7.65 as the pH of the 1:1000 (M/20,000) phosphate.   We would choose pH 7.6 if this were midway between the pH 7.0 and 7.8 indicators.   In fact, we note that the pH 7.65 chosen is also the midpoint of the readings pH 7.5, 7.65 and 7.8 given by the equally spaced pH 7.4, 7.6 and 7.8 indicators.   Hence a reading of pH 7.65 or possibly 7.67 is given accurately and quickly by the use of two of the shorter 3-pH phenol red series.   The 1:10,000 phosphate dilution (M/200,000) is brought still closer to pH 7.0 by the pH 7.0 superpure water and gives the readings pH 7.2 and 7.6 with the pH 7.0 and 7.8 phenol and cresol reds.   The average of the pH 7.2 and 7.6 is the pH 7.4 chosen which is also midway between the pH 7.0 and 7.8 indicators used and remains unchanged (isohydric) by the pH 7.4 indicator of the 9-point phenol and cresol red series.   It must therefore be nearly correct.   When the unknown solution is very dilute and the pH readings given by two of the 3-pH indicator series differ more than 0.2 to 0.4 pH or so much that the pH "chosen" as correct is in doubt, the two indicator solutions may be mixed in proper proportions from table 1 to give the "chosen" pH and this indicator added to the unknown solution should give

a final very accurate pH reading.   It is not safe to make linear interpolations to 0.1 pH on pH differences greater than about 0.4 pH because the pH scale is logarithmic, not linear, as Snyder (1928) has emphasized.   But if the research work involves constant tests of extremely dilute solutions or superpure water, it is much more convenient and accurate to make up the longer 9-pH indicator series and make the final pH measurement with that indicator solution most nearly isohydric with the pH given by the 3-pH series.   Tables 3, 4 and 5 contain a few heavily typed pH values "chosen" by the isohydric method as illustrations.

<div align="center">DEFINITIONS</div>

Before presenting or discussing the actual data it will be necessary to define certain terms which will appear in the text.

In discussing the data presented in the tables and curves, two terms will be used which may need definitions, viz., *"water-indicator effect"* and *"buffer-dilution effect."*   The first term is used to mean the combined effects of the water used (controlled mainly by the presence or the absence of CO_2) and of the indicator ions.   These effects are, obviously, to be seen only in the higher dilutions.   The second term is used to mean the effect produced by changes in ionization due to dilution of the buffer substances forming the basis of the solution or medium.   This effect is important in the lower dilutions and may be either in the acid or in the alkaline direction.   The chemical basis of buffer dilution is not yet well systematized with accurate data and need not be discussed here.[4]

When diluting with water containing CO_2 these two effects may be in opposite directions, as will be shown.   When, however, CO_2-free water is used, buffer-dilution and water-indicator effects may merge imperceptibly as we find ourselves dealing either primarily with the ionizing substances of the medium in the first part of the curve (lower dilutions) or with the ionization of the indicator itself as an important source of H or OH ions in the

[4] See Sörenson's table of pH values for different dilutions of glycocoll and asparagine given in Clark's Determination of H-ions, 1920 Ed., p. 30, and similar data on aniline hydrochloride given by Loomis and Acree, 1911.

TABLE 2

Dilution of acid potassium phthalate-HCl buffer brom phenol blue

pH OF INDICATOR	ELECTROMETRIC pH OF UNDILUTED BUFFER 0.05 M‡	DILUTION FACTORS							CO₂-FREE WATER*
		1:5 0.01 M	1:10 0.005 M	1:50 0.001 M	1:100 0.0005 M	1:200 0.00025 M	1:500 0.0001 M	1:1,000 0.00005 M	
		Colorimetric pH of diluted buffer							
3.4	3.75	3.77	3.80	4.0	4.1	4.2	4.35	4.45	4.6
3.6	3.75	3.77	3.80					4.45	4.6+
3.8	3.75	3.77	3.80					4.55	4.8+
4.0	3.75	3.77	3.80		4.15	4.3	4.5	4.60	4.8+
4.2	3.75	3.77	3.80					4.60	4.8+
4.4	3.75	3.77	3.80					4.70	4.8+
4.6	3.75	3.77	3.80	4.0	4.2	4.35	4.6	4.80	4.8+
									AIR-CO₂ EQUILIBRIUM WATER†
3.4	3.75	3.77	3.8	4.0	4.1	4.2	4.35	4.45	4.6
3.6	3.75	3.77	3.8	4.0	4.1	4.25	4.4	4.50	4.6+
3.8	3.75	3.77	3.8	4.0	4.1	4.25	4.42	4.52	4.8
4.0	3.75	3.77	3.8	4.0	4.1	4.25	4.45	4.55	4.8+
4.2	3.75	3.77	3.8	4.0	4.1	4.25	4.50	4.55	4.8+
4.4	3.75	3.77	3.8	4.0	4.1	4.30	4.55	4.60	4.8+
4.6	3.75	3.77	3.8	4.0	4.15	4.35	4.60	4.6+	4.8+
									pH 5.0 WATER DIRECT FROM STILL
3.4	3.75	3.77	3.8−	4.0	4.1	4.25	4.35	4.45	4.6
3.6	3.75	3.77							4.6+†
3.8	3.75	3.77							4.8
4.0	3.75	3.77			4.15	4.35	4.45	4.6	4.8+
4.2	3.75	3.77							4.8+
4.4	3.75	3.77							4.8+
4.6	3.75	3.77	3.8−	4.0	4.2	4.4	4.6	4.7	4.8+

* pH 7.0 with brom thymol blue (7.0).

† pH 5.65 with brom cresol purple (5.7).

‡ In this and all succeeding tables the electrometric readings were made on the buffer without the presence of an indicator. Where the colorimetric value is repeated throughout an entire column actual readings were made only at the beginning and end. This applies to all succeeding tables as well.

last part (covering the higher dilutions). In either case the dilution effect is probably of the same nature from a chemical viewpoint; that is, it is due to ionization and hydrolysis of systems of weak acids or bases and their salts.
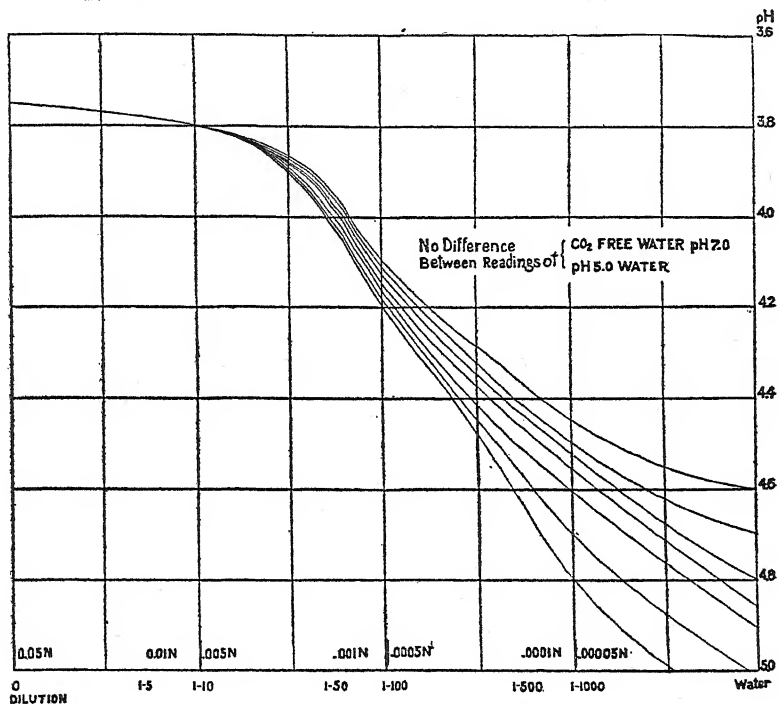
FIG. 1. RELATION OF pH VALUES TO DILUTION OF PHTHALATE-HCl BUFFER (ELEC. pH 3.75) WHEN TESTED WITH BROM PHENOL BLUE IN ADJUSTED SERIES (pH 3.4 TO 4.6 IN INTERVALS OF 0.2 pH)

In studying the tables and charts the reader must keep in mind that the buffer-dilution curves would be slightly more alkaline if the points were determined electrometrically as will be demonstrated in the paper on *salt errors* now under way. Hence in all previous work the errors due to CO_2 and indicators are even more misleading than they appear to be from the colorimetric data.

DISCUSSION OF TABLES AND CURVES

Dilution of acid potassium phthalate-HCl buffer of pH 3.75
(brom phenol blue)

In table 2 (fig. 1) are presented the data obtained on dilution of an acid-potassium phthalate-HCl buffer mixture (0.05M).

Differences in pH due to dilution are apparent throughout, grading evenly from 1:10 where they are first appreciable to 1:1000 where the readings are barely distinguishable from those of water itself. This gradual increase in pH in the upper part of the curves as far as 1 part in 50, falls without doubt under the category of *buffer-dilution effect.* In about that region of the curves representing a dilution of 1 part in 100 (0.0005 M) the *buffer dilution and water-indicator effects* merge and we have as a result smooth rapidly descending curves. It will be seen that all the readings obtained when the indicator, whatever its adjustment, was introduced into water alone (see last column) are either near the extreme alkaline end of the range of brom phenol blue (pH 4.8) or beyond it.

That practically no differences in pH are found between readings made with pH 7.0, pH 5.7 and pH 5.0 water, provided the adjustment of the indicator solution is the same, is due no doubt to the fact that formation of hydrogen ions from the ionization of the indicator acid is of a greater order than that from the ionization of the acid formed by the amount of dissolved CO_2 present, even in pH 5.0 water.

Differences due to the adjustment of the indicator are slight and do not become apparent until a dilution of 1 part in 100 is reached. Even in the higher dilutions the range of values obtained with the adjusted series of indicator solutions is much more restricted than that of the readings made with other indicator series at the same dilutions (see other charts). This is due to the fact that the medium being diluted is a comparatively strong acid buffer which over-balances the lower acidities and concentrations of this indicator at all its pH values.

Colorimetric readings agree with the electrometric reading on the undiluted buffer within an error of 0.05 pH when the buffer is

TABLE 3

Dilution of 1 per cent peptone beef infusion bouillon

Brom cresol purple

pH OF INDICATOR	ELECTROMETRIC pH OF UNDILUTED MEDIUM	COLORIMETRIC pH OF UNDILUTED MEDIUM	DILUTION FACTORS						CO$_2$-FREE WATER*
			1:5	1:10	1:50	1:100	1:500	1:1,000	
			Colorimetric pH of diluted medium						
5.2	6.05	6.0+	6.0+	6.0	6.0	5.95*	5.85	5.80	5.70
5.4	6.05	6.0+	6.0+	6.0			5.85	5.80	5.75
5.6	6.05	6.0+	6.0+	6.0			5.95	5.90	5.9−
5.8	6.05	6.0+	6.0+	6.0			6.0−	5.95	6.0
6.0	6.05	6.0+	6.0+	6.0			6.05	6.00	6.05
6.2	6.05	6.0+	6.0+	6.0			6.10	6.10	6.3−
6.4	6.05	6.0+	6.0+	6.0			6.2−	6.20	6.4
6.6	6.05	6.0+	6.0+	6.0	6.05	.6.1	6.25	6.30	6.6−
									AIR-CO$_2$ EQUILIBRIUM WATER†
5.2	6.05	6.0+	6.0	6.0	5.95	5.90	5.75	5.6	5.45
5.4	6.05	6.0+	6.0	6.0			5.8−	5.6+	5.50
5.6	6.05	6.0+	6.0	6.0			5.80	5.7	5.60
5.8	6.05	6.0+	6.0	6.0			5.85	5.75	5.65
6.0	6.05	6.0+	6.0	6.0			5.9	5.8−	5.75
6.2	6.05	6.0+	6.0	6.0			5.95	5.85	5.85
6.4	6.05	6.0+	6.0	6.0			6.00	5.9	5.90
6.6	6.05	6.0+	6.0	6.0	6.0	6.05	6.05	6.0	6.0
									SAME WATER CO$_2$ FROM TANK‡
5.2	6.05	6.0+	6.0	5.9	5.6	5.4	5.2	5.2−	5.0
5.4	6.05	6.0+	6.0	5.9					5.0
5.6	6.05	6.0+	6.0	5.9					5.0
5.8	6.05	6.0+	6.0	5.9					5.0
6.0	6.05	6.0+	6.0	5.9					5.0
6.2	6.05	6.0+	6.0	5.9					5.0
6.4	6.05	6.0+	6.0	5.9					5.0
6.6	6.05	6.0+	6.0	5.9	5.6	5.4	5.2+	5.1	5.0

* pH 7.0 with brom thymol blue (7.0).

† Note that the point of agreement between the pH of adjusted indicators and the series of pH values for water in the last column is at 5.6 instead of 5.7. This may have been due to impurity of the air in the room since the fresh air was not always kept bubbling through the dilution water.

‡ pH 5.0 brom cresol purple (5.2); pH 5.0 methyl red (5.0). CO$_2$ was not measured in this case and the water was made a little too acid when introducing CO$_2$ directly from the tank. The actual pH was probably 4.8 instead of 5.0.
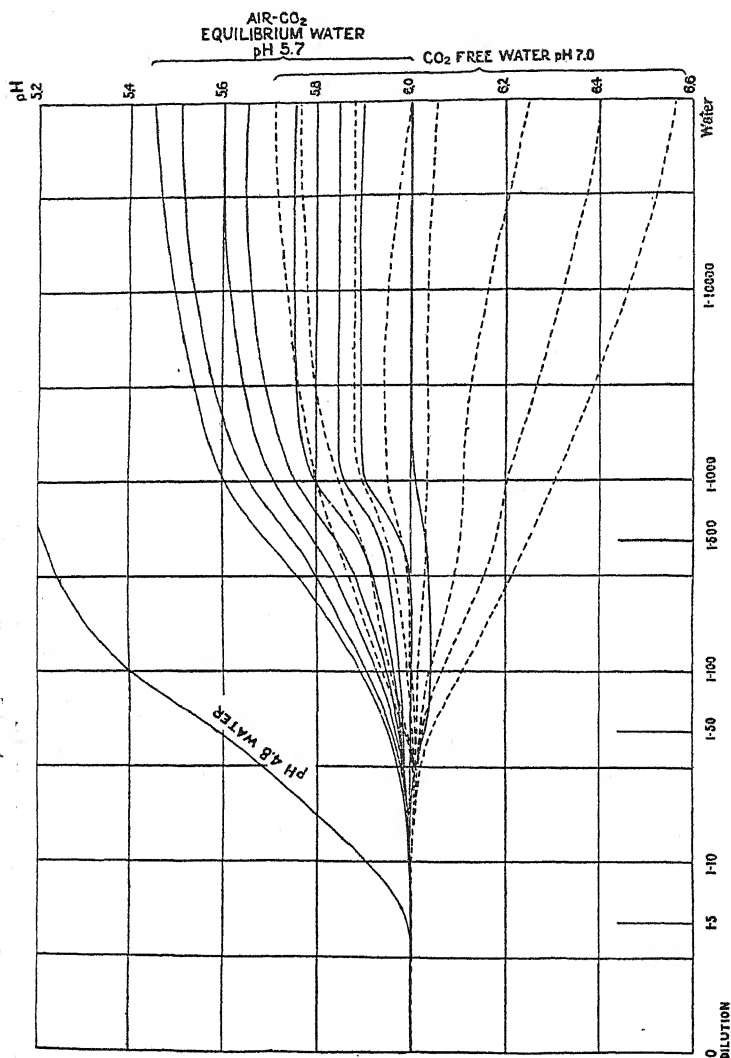
FIG. 2. RELATION OF pH VALUES TO DILUTION OF 1 PER CENT PEPTONE BEEF INFUSION BOUILLON (ELEC. pH 6.05) WHEN TESTED WITH BROM CRESOL PURPLE IN ADJUSTED SERIES (pH 5.2 TO 6 6 IN INTERVALS OF 0.2 pH)

not diluted further than 1 part in 10. At 1 part in 50 the difference reaches 0.25 pH and rapidly increases as dilution proceeds.

Dilution of 1 per cent peptone, beef infusion bouillon (brom cresol purple)

In table 3 (fig. 2) are presented dilution data for 1 per cent peptone beef infusion bouillon.

There are no *changes in pH due to dilution* worthy of note until 1 part in 50 is reached and then these are very slight except with pH 4.8 water in which case the high CO_2 *content* deflects the curves to the extent of 0.1 pH at a dilution of 1:10 and causes all the curves to fall into a single line, a very good illustration of the buffering effect of CO_2 in water. Likewise, *the differences due to indicator adjustment* seen when the other waters were used are here wiped out. In contrast to the brom phenol blue series in table 2 (fig. 1), where the indicator acid is a stronger factor than the CO_2 effect, here the effect of CO_2 out-balances the effect of the brom cresol purple indicator regardless of its pH; in other words, the CO_2 in solution forms a stronger acid than the indicator acid in any part of the adjusted series.

On the other hand *the effect of CO_2* is scarcely appreciable in readings made with pH 5.7 water until a dilution of 1 part in 500 is reached where the greatest difference between readings made with pH 5.7 and CO_2-free (pH 7.0) water was 0.2 pH and even at a dilution of 1:1000 the error is only 0.3 pH at the extreme alkaline end of the indicator range (pH 6.6). At the mid-point of the indicator series this type of error does not exceed 0.15 pH at a dilution of 1:500 or 0.2 pH at 1:1000.

There is almost exact *agreement between colorimetric readings and the electrometric reading* on the undiluted medium when the dilution does not exceed 1:10 with the exception of those made with pH 4.8 water. In the case of CO_2-free water, errors are not greater than 0.1 pH even at a dilution of 1:100, and then only at the extremes of the indicator series. At its mid-point (pH 6.0) the values recorded are practically identical throughout the

dilution series and agree with the electrometric reading; this is obviously because the pH of the indicator used (6.0) almost exactly coincides with that of the medium under test (6.05) and because the pH of a brom cresol purple solution adjusted to pH 6.0 was changed very slightly (6.0 to 6.05) when introduced into CO_2-free water (see last column on the right).

Were the solution being measured of a H-ion concentration near the acid end of the range of brom cresol purple, for example pH 5.2 to 5.4, readings made with pH 5.7 or even pH 5.0 water would agree more nearly with the true pH than those made with pH 7.0 water. Seldom in ordinary practice would every condition be found ideal, as in the case just cited where dilution did not greatly effect the pH value in any part of the series from the undiluted medium all the way down to water itself. The same condition may be observed at some one point in all of the other charts.

Dilution of Fermi's solution[5] (brom thymol blue)

Table 4 (fig. 3) embodies dilution data for Fermi's solution—a much used, highly buffered, synthetic medium—and exemplifies in a striking way all the principles previously discussed.

In addition figure 3 illustrates well the fact that when a medium, the reaction of which is in the region of neutrality, is diluted with water containing CO_2 the initial changes may be in the opposite direction from those which occur on further dilution. Thus it will be seen that the curves dip to the extent of 0.15 pH at 1 part in 5 due to *buffer-dilution* and that later most of them rise again but at markedly different rates according to the amount of CO_2 in the water or the pH of the indicator used.

Another fact which stands out in this table is the extreme to which dilution may be carried (1 part in 100,000) and still show

[5] Distilled water..1,000 cc.
MgSO$_4$.. 0.2 gram
KH$_2$PO$_4$.. 1.0 gram
(NH$_4$)$_2$HPO$_4$...10.0 grams
Glycerine.. 45.0 cc.
This solution is 0.083 m with respect to the phosphate buffer. Since the MgSO$_4$ does not affect the buffer action above pH 7.0 it was ignored in the calculation.

TABLE 4

Dilution of Fermi's solution

Brom thymol blue

pH OF INDI-CATOR	ELECTRO-METRIC pH OF UNDI-LUTED BUFFER	COLORI-METRIC pH OF UNDI-LUTED BUFFER	DILUTION FACTORS								CO₂-FREE WATER*
			1:5 0.0166 M	1:10 0.0083 M	1:50 0.00166 M	1:100 0.00083 M	1:500 0.000166 M	1:1,000 0.000083 M	1:10,000 0.0000083 M	1:100,000 0.0000083 M	
			Colorimetric pH of diluted medium								
6.2	7.00	7.0+	7.15	7.15	7.2	7.15	7.10	7.02	6.95	6.6	6.4
6.4	7.00	7.0+	7.15	7.15	7.2	7.15			7.00	6.7	6.5+
6.6	7.00	7.0+	7.15	7.15	7.2	7.15			7.02	6.75	6.75
6.8	7.00	7.0+	7.15	7.15	7.2	7.15			7.05	6.85	6.85
7.0	7.00	7.0+	7.15	7.15	7.2	7.15			7.10	7.05	7.05
7.2	7.00	7.0+	7.15	7.15	7.2	7.15			7.15	7.2	7.2+
7.4	7.00	7.0+	7.15	7.15	7 2	7.15			7.17	7.4−	7.4−
7.6	7.00	7.0+	7.15	7.15	7.2	7.15	7.15	7.15	7.20	7.4	7.5
											AIR-CO₂ EQUILIBRIUM WATER†
6.2	7.00	7.0+	7.15	7.15	7.15	7.15	7.05	6.85	6.1		6.0−
6.4	7.00	7.0+	7.15	7.15	7.15	7.15			6.1		6.0−
6.6	7.00	7.0+	7.15	7.15	7.15	7.15			6.2		6.0
6.8	7.00	7.0+	7.15	7.15	7.15	7.15			6.25		6.0
7.0	7.00	7.0+	7.15	7.15	7.15	7.15			6.3		6.1
7.2	7.00	7.0+	7.15	7.15	7.15	7.15			6.4		6.2
7.4	7.00	7.0+	7.15	7.15	7.15	7.15			6.5		6.3+
7.6	7.00	7.0+	7.15	7.15	7.15	7.15	7.15	7.00	6.6		6.35
											pH 5.0 WATER‡
6.2	7.00	7.0+	7.15	7.15	7.15	7.05	6.5	6.0	6.0−	Full acid color	Full acid color
6.4	7.00	7.0+	7.15	7.15	7.15	7.05				Full acid color	Full acid color
6.6	7.00	7.0+	7.15	7.15	7.15	7.05				Full acid color	Full acid color
6.8	7.00	7.0+	7.15	7.15	7.15	7.05				Full acid color	Full acid color
7.0	7.00	7.0+	7.15	7.15	7.17	7.05				Full acid color	Full acid color
7.2	7.00	7.0+	7.15	7.15	7.15	7.05				Full acid color	Full acid color
7.4	7.00	7.0+	7.15	7.15	7.15	7.05				Full acid color	Full acid color
7.6	7.00	7.0+	7.15	7.15	7.15	7.10	6.6	6.2	6.0	Full acid color	Full acid color

* pH 7.05 with B.T.B. (7.0).

† pH 5.7 with B.C.P. (5.7).

‡ Direct from still.
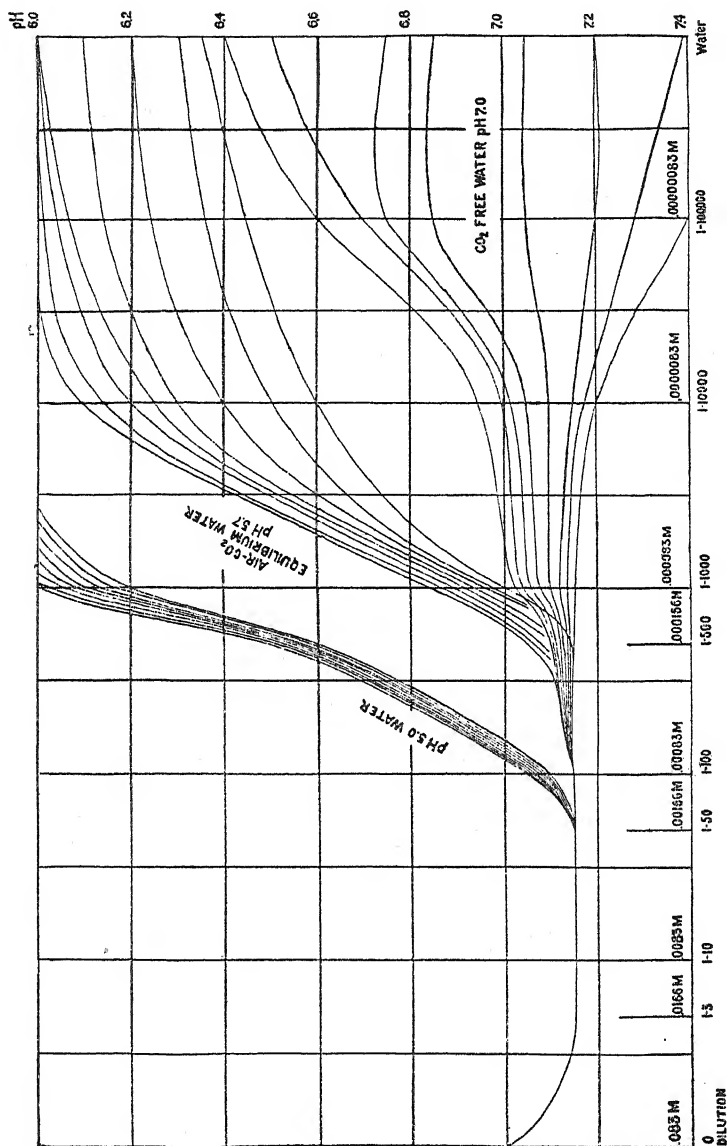
FIG. 3. RELATION OF pH VALUES TO DILUTION OF FERMI'S SOLUTION (ELEC. pH 7.0) WHEN TESTED WITH BROM
THYMOL BLUE IN ADJUSTED SERIES (pH 6.2 TO 7.6 IN INTERVALS OF 0.2 pH)

evidence in the readings of the presence of the medium.   This
is true especially where CO_2-free water is used, and to a slightly

TABLE 5

Dilution of acid potassium phosphate-NaOH buffer

Phenol red, cresol red.   Data with CO_2-free water

INDICATOR	pH OF INDICA- TOR	ELEC. pH OF UN- DILUTED BUFFER	DILUTION FACTORS					CO₂ FREE WATER
			1:5 0.01M	1:10 0.005M	1:100 0.0005M	1:1000 0.00005M	1:10,000 0.000005M	
			Colorimetric pH of diluted buffers					
Phenol red........	7.0	7.82	7.8+	7.8+	7.8	7.5	7.2	7.0
Cresol red........		7.82	7.8+	7.8+	7.8	7.5	7.25	7.0−
Phenol red........	7.2	7.82	7.8+	7.8+	7.8	(7.55)*	7.45	7.2
Cresol red........		7.82	7.8+	7.8+	7.8	(7.55)	7.35	7.2−
Phenol red........	7.4	7.82	7.8+	7.8+	7.8	7.6	7.4	7.35
Cresol red........		7.82	7.8+	7.8+	7.8	7.6	**7.4**	7.3
Phenol red....˙....	7.6	7.82	7.8+	7.8+	7.8	(7.65)	7.5	7.45
Cresol red........		7.82	7.8+	7.8+	7.8	**(7.65)**	7.5	7.4
Phenol red........	7.8	7.82	7.8+	7.8+	7.8+	7.7	7.6−	7.6
Cresol red........		7.82	7.8+	7.8+	7.8	7.7	7.6	7.6
Phenol red........	8.0	7.82	7.8+	7.8+	7.8	(7.75)	(7.7)	7.8
Cresol red........		7.82	7.8+	7.8+	7.8+	(7.75)	7.8	7.8
Phenol red........	8.2	7.82	7.8+	7.8+	7.8	7.8	7.8	7.9
Cresol red........		7.82	7.8+	7.8+	7.8	7.8	(7.8)	7.9
Phenol red........	8.4	7.82	7.8+	7.8+	7.8	(7.85)	7.9	8.0
Cresol red........		7.82	7.8+	7.8+	7.8	(7.85)	7.85	7.95
Phenol red........	8.6	7.82	7.8+	7.8+	7.85	7.9	7.95	8.1
Cresol red........		7.82	7.8+	7.8+	7.8+	7.9	7.9	8.0

* Figures in parentheses were interpolated.

less degree with air-CO_2 equilibrium water.   In the case of pH
5.0 water the buffering power of the CO_2 practically obliterates
the effect of the medium in the highest dilutions.

TABLE 6

Dilution of acid potassium phosphate-NaOH buffer

Phenol red, cresol red

INDICATOR	pH OF INDICATOR	ELEC. pH OF UNDILUTED BUFFER	DILUTION FACTORS						AIR-CO₂ EQUILIBRIUM WATER*
			1:5	1:10	1:50	1:100	1:500	1:1,000	
			Colorimetric pH of diluted buffer						
			Data with air-CO₂ equilibrium water						
Phenol red.........	7.0	7.82	7.8	7.8	7.75 7.65 7.65 7.65	7.6 7.55 7.55 7.55	7.2 7.15 7.1 7.05	7.0— 5 minutes 6.8—30 minutes 6.8— 1 hour 6.7—1½ hours	Full acid color
Cresol red.........	7.0	7.82	7.8	7.8	7.75	7.6	7.2 7.3	7.0— 5 minutes 7.1— 5 minutes	Full acid color
Phenol red.........	7.8	7.82	7.8	7.8			7.2 7.15 7.15	30 minutes 1 hour 1½ hours	
Cresol red.........	7.8	7.82	7.8	7.8	7.8 7.7 7.7	7.65 7.6 7.6	7.4 7.25 7.2	7.1— 5 minutes 7.2— 5 minutes	Full acid color
Phenol red.........	8.6	7.82	7.8	7.8	7.7	7.6—	7.2—	7.0—30 minutes 7.0— 1 hour 6.9—1½ hours	
Cresol red.........	8.6	7.82	7.8	7.8	7.8	7.65	7.4	7.2— 5 minutes	

Data with pH 5.0 water

								pH 5.0 WATER†
Phenol red........	7.0	7.82	7.8	7.8	7.50	7.25	Full acid color	Full acid color
Cresol red........			7.8	7.8	7.50	7.25	Full acid color	Full acid color
Phenol red........	7.8	7.82	7.8	7.8	7.57	7.3	Full acid color	Full acid color
Cresol red........					7.55	7.3		Full acid color
Phenol red........	8.6	7.82	7.8	7.8	7.6	7.35	Full acid color	Full acid color
Cresol red........			7.8	7.8	7.6	7.35		Full acid color

* pH 5.7 with B.C.P. 5.7.

† One cubic centimeter water saturated with CO_2 per 200 cc. CO_2-free water.   pH 5.0 B.C.P. (5.0).   pH 5.2 M.R. (5.0).

Dilution of acid potassium phosphate-NaOH buffer (Phenol red, cresol red)

The data given in tables 5 and 6 (fig. 4), show more strikingly than do any of the other experiments the *effect of CO_2 on the readings* because of the alkaline range covered. The errors are nearly all in the acid direction and increase in direct proportion to
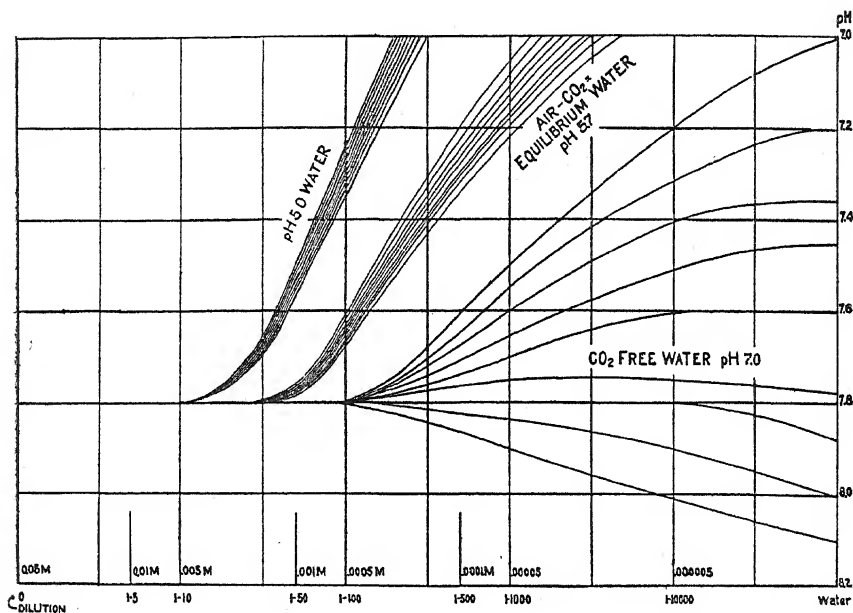
FIG. 4. RELATION OF pH VALUES TO DILUTION OF PHOSPHATE-NaOH BUFFER (ELEC. pH 7.82) WHEN TESTED WITH PHENOL RED VS. CRESOL RED IN ADJUSTED SERIES (pH 7.0 TO 8.6 IN INTERVALS OF 0.2 pH)

the amount of CO_2 present. In table 5 where the readings were made under CO_2-free conditions the deflections from the true pH due to dilution are slight even at 1:1000, the greatest error being 0.3 pH at the extreme acid end of the indicator series, while in table 6 where pH 5.7 water was used they begin to be noticeable at 1:100, and in the case of pH 5.0 water at 1:50. Even with regard to the alkaline buffer, however, the rule holds that errors

due either to CO_2 or to adjustment of the indicator are negligible at a dilution of 1 part in 10 or less.

The outstanding point in table 6 is the *slow action of CO_2 on the readings made with pH 5.7 water*. The change in the tubes was so apparent that it was necessary to wait several minutes before taking the first readings. After five minutes the change was more gradual. A study of the figures shows that the greatest change occurred in the 1:1000 dilution where the difference between the five-minute and one-and-one-half-hour readings was 0.3 pH. At 1:500 it was 0.15 to 0.25 pH in the same length of time, and at 1:50 only 0.1. It is also noteworthy that equilibrium was reached sooner in the lower dilutions. The experiment shows how unsatisfactory it is to work with very dilute solutions of an alkaline medium in the presence of CO_2. When pH 5.0 water was used the CO_2, present in larger amount, acted so rapidly that it was not necessary to wait longer than a minute before taking readings.

The differences due to indicator adjustment are not great, except in the highest dilutions of the CO_2-free series.

Good agreement was obtained between the readings made with the two indicators used, phenol red and cresol red, as also *between colorimetric readings and the electrometric reading* on the undiluted medium, under all conditions, at dilutions lower than and including 1 part in 10, and even at a dilution of 1 part in 100 under CO_2-free conditions; the last mentioned fact shows the strong buffering power of the phosphate mixture.

Dilution of 1 per cent peptone, 1 per cent glucose medium with redistilled water vs. ordinary distilled water (brom thymol blue)

Table 7 (fig. 5) records an experiment which was devised to show what influence, if any, the *presence of small quantities of sprayed-over alkaline salts in the ordinary distilled water* would have on the colorimetric pH of diluted media and what weight this influence would have as compared with the effect of varying amounts of CO_2 in the water. Accordingly redistilled water (pH 7.05 under CO_2-free conditions) was used in comparison with

Dilution of 1 per cent peptone-1 per cent glucose medium

Brom thymol blue

pH OF INDICATOR	COLORIMETRIC pH OF UNDILUTED MEDIUM	DILUTION FACTORS					REDISTILLED WATER CO₂-FREE
		1:5	1:10	1:50	1:100	1:500	
		Colorimetric pH of diluted medium					
6.2	6.5	6.5	6.4	6.4	6.4	6.4	6.4
7.0	(6.5)	(6.5)	(6.5)	6.6	6.7	6.9	7.05
7.6	6.5	6.6−	6.6	6.7	6.9	7.3	7.6
							ORDINARY DISTILLED WATER CO₂-FREE
6.2	6.5	6.5	6.4+	6.5	6.6	6.8	7.0
7.0	(6.5)	(6.5)	(6.5)	6.65	6.85	7.2	7.55
7.6	6.5	6.6−	6.6	6.8	7.0	7.45	7.6+
							REDISTILLED WATER: AIR CO₂ EQUILIBRIUM*
6.2	(6.5)	6.4	6.3	6.0			6.0−
7.0	(6.5)	6.4	6.4−	6.15			6.0−
7.6	(6.5)	6.4+	6.4	6.3			6.1
							ORDINARY DISTILLED WATER: AIR-CO₂ EQUILIBRIUM†
6.2	(6.5)	(6.4)	6.3	6.0+			6.0−
7.0	(6.5)	(6.4)	6.3	6.2			6.1
7.6	(6.5)	(6.4+)	6.4	6.3+			6.3
							REDISTILLED WATER (pH 5.0)‡
6.2	6.5	6.2	6.0				6.0−
7.0	(6.5)						
7.6	6.5	6.2+	6.1				6.0−
							ORDINARY DISTILLED WATER (pH 5.1)‡
6.2	6.5	6.2	6.0				6.0−
7.0	(6.5)						
7.6	6.5	6.2+	6.1				6.0

* pH 5.5 with B.C.P. (5.6). The CO₂ content was a little high because this equilibrium was obtained by running ordinary air through pH 5.0 water without heating. It would require a long time to entirely drive out the extra quantity of CO₂ in this way.

† pH 5.8 with B.C.P. (5.6). The difference between 5.5 and 5.8 was due to salt content.

‡ pH 5.0 to 5.1 with brom cresol purple (5.0) prepared in each case by adding 1 cc. of redistilled water saturated with CO₂ to 200 cc. of the dilution water.

Figures in parentheses interpolated,—all others actual readings.
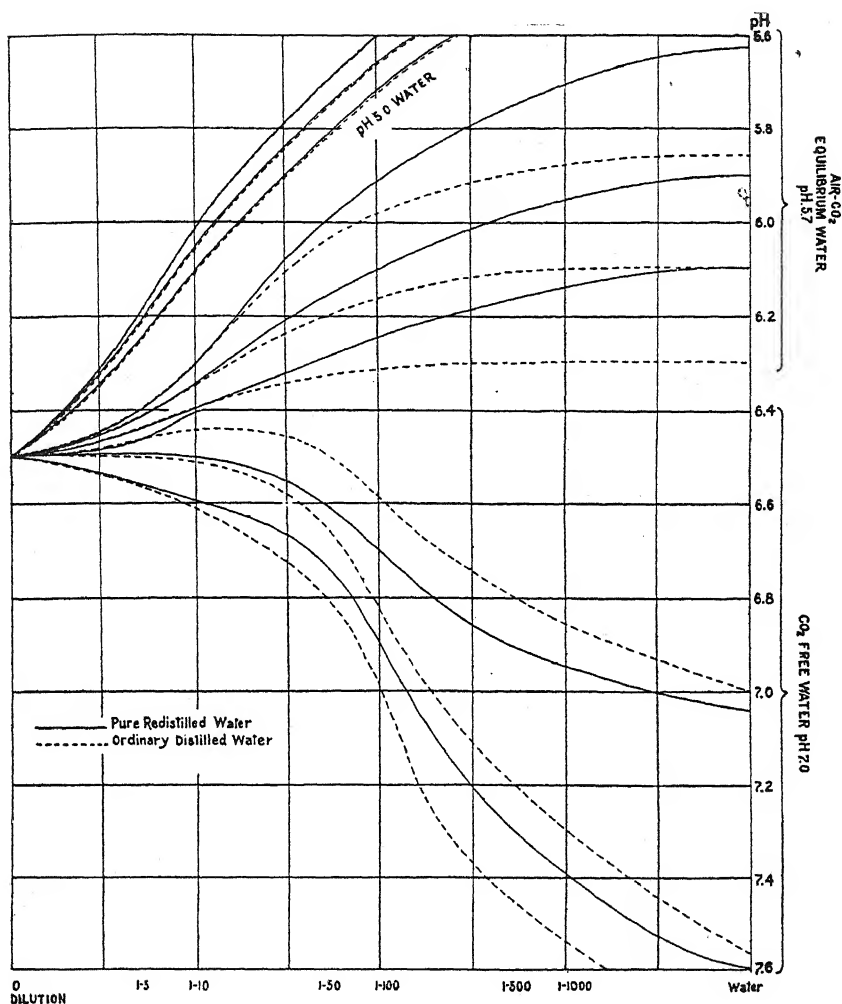
FIG. 5. RELATION OF pH VALUES TO DILUTION OF 1 PER CENT PEPTONE-1 PER CENT GLUCOSE MEDIUM WHEN TESTED WITH BROM THYMOL BLUE IN ADJUSTED SERIES (pH 6.2, 7.0, 7.6)

a sample of ordinary distilled water from a commercial still, testing pH 7.55 with brom thymol blue (7.0) and pH 8.0 with phenol red (pH 8.0) when aerated with CO_2-free air, and testing pH 5.1 with brom cresol purple (5.0) as collected. The medium chosen was a comparatively weakly buffered one, viz., 1 per cent peptone, 1 per cent glucose solution.

The chart brings out two important points (1) that the pH of the diluted medium is affected to a far greater extent by the CO_2 content of the water than by its salt content and (2) that differences in pH due to variation in CO_2 content and also to a slight extent to indicator adjustment occur even in the lowest dilutions in contrast to the more heavily buffered media.

When both of the waters are supercharged with CO_2 (pH 5.0 to 5.1) the CO_2 effect so outweighs the salt content effect that the latter is obliterated. Note that the solid lines and the dotted lines follow the same path. At air-CO_2 equilibrium the differences between readings made with redistilled and ordinary distilled water are barely apparent at a dilution of 1 part in 50 and do not exceed 0.2 pH even when there is no buffer present (see readings of indicators in water alone). On the other hand, under CO_2-free conditions, the differences in pH due to the salt content of the ordinary distilled water are considerable in the higher dilutions (0.1 to 0.40 pH) and very marked (as high as 0.5 to 0.6 pH) when the two waters are compared alone without the presence of buffer. It is reassuring however to find that water with a high enough buffer salt content (pH 8.0) to register pH 7.55 with brom thymol blue adjusted to pH 7.0, does not give readings different from those obtained with pH 7.0 water at dilutions of 1 part in 5 or 1 part in 10.

It is noteworthy that a reading of pH 7.0 was obtained on this water (see last column on the right) with an indicator solution adjusted to pH 6.2, whereas a reading of pH 7.55 was obtained with the indicator adjusted to pH 7.0. This well illustrates the point that a reading of pH 7.0 on water can not be accepted as the true one unless the indicator with which it was obtained was adjusted to pH 7.0. The reading of pH 7.0 with the pH 6.2 indicator can easily be explained as being due to a partial neutrali-

zation of the indicator acid by the sprayed-over alkaline buffer salts in the CO_2-freed ordinary distilled water (pH 8.0).   Hence ordinary distilled waters containing enough CO_2 to test pH 5.0 may be treated with pure air to remove the CO_2 just sufficiently to neutralize the sprayed-over alkaline salts and give pH 7.0 with pH 7.0 brom thymol blue.   But such waters are variable and unstable, become alkaline when boiled or aerated with CO_2-free air, exert a noticeable buffer effect when used to dilute media and buffers, give pH readings with adjusted bromthymol blue which are misleading regarding the purity of the water, and generally have specific conductivities above 2×10^{-6} arising from the sprayed-over buffer and neutral salts.   But double distilled CO_2-free water of pH 7.0 and a specific conductivity less than 0.5×10^{-6} is easily prepared, stored and used by means of the stills and methods described herein, is free from all these objections, and is earnestly recommended for general use with the isohydric indicator methods in all colorimetric H-ion investigations.

<div align="center">RECOMMENDATIONS</div>

The following *recommendations* are offered as a result of these investigations:

1. All *glass ware* used must be Pyrex or non-sol in character and scrupulously cleaned by such methods as are described in the early part of this paper under *Technic Sec. 1, Glassware.*

2. The *method of filling tubes* for tests and of introducing the indicator must be uniform, and designed to prevent or minimize changes in CO_2 equilibria.   The method found most satisfactory by the authors is described *under Technic, Secs. 5–6.*

3. In colorimetric work with very dilute solutions the *purity of the water* used and hence its preparation by a reliable method, designed to prevent the absorption of *solids* from the apparatus as well as the carrying over of *buffer sprays*, is extremely important. The main features of our table still (to be further described in a separate paper) are indicated *under Technic, Sec. 2, Water.*

The presence of varying quantities of CO_2 in distilled water has been found to be a far more important factor affecting pH

measurements than the presence of other impurities usually found, although the latter must not be ignored.   Hence measures must be taken either to *control the amount of CO_2 in the dilution* water *or to eliminate* it entirely.

a. Consistent, though not always accurate results may be obtained in dilute solutions by using water which has reached equilibrium with the CO_2 in the air (pH 5.7) but water containing even so small a quantity of CO_2 will measurably affect readings when working in the ranges more alkaline than pH 5.7 (air-CO_2 equilibrium).   Generally speaking, however, air-equilibrium water will not seriously vitiate determinations made within the range of brom cresol purple (pH 5.0 to pH 6.6).

b. Water freshly collected from ordinary stills is commonly supercharged with CO_2, having a pH value of approximately 5.0 and cannot be used for making determinations in very dilute solutions more alkaline than pH 5.0 without affecting the readings.

c. In the ranges covered by the indicators brom thymol blue (pH 6.2 to 7.6), phenol red (pH 6.8 to 8.4) and cresol red (pH 7.2 to 8.8) it is essential to work under CO_2-free conditions in order to prevent serious errors in determining colorimetrically the pH of weakly buffered solutions, especially toward the alkaline ends of their ranges.   A simple method of removing CO_2 from water is described *under Technic, Sec. 2, Water.*

4. For accurate work with solutions of the usual unknown concentrations (0.1 to 0.001 M), it is advisable to *adjust the indicator to the mid-point* of its useful range.   When only one stock solution of each indicator is made up for *general tests,* the usual practice of today, we have used since January, 1924, and recommend 1.5 mols of alkali with the exceptions noted, or specifically such amount of acid or alkali as is shown in table 1 to be necessary to bring the pH value of the single stock indicator solution close to the mid-point.   This procedure must on the average, except in the most dilute solutions, balance up the small effect of the indicator itself on the pH of the mixture measured.   The slight error which may still occur in very weakly buffered solutions at the ends of the useful indicator range is

annulled by taking the average of the slightly too high and too low readings found by using overlapping indicator ranges in a test.

5. When dealing with *an extremely dilute solution* the concentration of which is of an order only slightly greater or less than that of the indicator itself (0.0001 M and less), approximate results may be obtained by testing the unknown with three solutions of the indicator adjusted at the lowest, midpoint and highest useful pH values, or still better with a series of solutions of the same indicator graded in steps of 0.1 to 0.2 pH, and determining the point or range at which there is coincidence between the pH as read and that of the indicator solution used. A simple practical *method of adjusting indicators to graded pH values* is described under *Technic, Sec. 3*. No successful method has yet been found for stabilizing these delicately adjusted solutions but it is essential that they be made up in scrupulously clean containers and stored in pyrex or non-sol bottles or flasks to prevent change due to contact with soft glass as has been noted also by other workers. A more detailed account of the iso-indicator method and its applications will be given in another paper now in preparation by the authors entitled, "A new method of determining the purity of water by means of indicators." This paper will also contain a complete study of CO_2 equilibria in water and methods of determining the pH of water under all laboratory conditions.

GENERAL CONCLUSIONS

1. A study of the dilution curves for the different buffers and media investigated reveals three types of variation from the true pH value as electrometrically determined for each solution in the undiluted state: (1) buffer-dilution effects; (2) changes caused by the varying CO_2 content and alkaline impurities of the water used; and (3) variations caused by the differences in the pH values of the indicator solutions. *Buffer dilution* and *water-indicator* effects may be in the same direction, as in figure 1, or in opposite directions, as in figure 3 where the initial dilutions cause an alkaline change followed by more or

less marked increases in acidity upon further dilution due to varying amounts of CO_2 in the water and the pH of the indicators used.

2. *The effect of CO_2* in the water is not marked as a rule until a dilution of 1 part in 50 is reached. As dilution is increased the effect of CO_2 rapidly becomes more marked, especially in the case of pH 5.0 water often carrying the curves beyond the range of the indicators used. The curves made with air-CO_2 equilibrium (pH 5.7) water naturally fall in a position intermediate between those made with pH 5.0 water and with CO_2-free (pH 7.0) water.

3. In all the charts with the exception of Chart 1, it is very noticeable that CO_2 acts not only as an *acid*, lowering the pH values markedly in the more dilute solutions, but that it also acts as a *buffer*, causing the readings made with indicator solutions adjusted in graded series to *fall closer together* than those made under CO_2-free conditions. This phenomenon increases with the amount of CO_2 present. The effect produced by the curves is like that of the opening or closing of a fan and sometimes of a considerable shifting of the fan to a different position on the chart (figures 3 and 4 for the buffers of higher pH ranges).

4. *The adjustment of the indicator solution* itself does not begin to cause noticeable variations in readings until a dilution of 1 part in 100 is reached in the organic media (figs. 2 and 5) or molalities of 0.0001 to 0.000083 in the synthetic media (figs. 3 and 4) and it was necessary to dilute the latter solutions to 0.00005 M or 0.0000083 M before the adjustment of the indicator became a very important factor in controlling the pH.

5. In all the charts one general principle stands out, viz., that barring the comparatively slight initial variations due to buffer dilution, the readings obtained with an indicator solution adjusted to a given pH value approach closer and closer, as dilution proceeds, to the value registered by that indicator solution in the same water without the presence of the buffer (see values recorded at the extreme right of every chart).

6. A paper now in preparation dealing with *salt errors* in relation to dilution will bring out more clearly the value of the technic

described in the present article in making it possible to separate CO_2 and indicator effects from true salt effects. The statement should be made here that the *electrometric* data show rather marked increases in pH due to buffer dilution even in the first dilutions (1:5 and 1:10) in both buffers and media. This same change in pH measured *colorimetrically* is greatly lessened or entirely annulled by the fact that the salt errors of the indicators are nearly always in the acid direction; hence *colorimetrically* there often appears to be little or no change in pH in the initial dilutions, a fact which has proved to be useful in practice rather than a detriment when turbid or colored solutions are diluted to get the pH values.

7. Although the dilutions showing variations due to CO_2 content of water and adjustment of indicator solutions are higher than those recommended for ordinary colorimetric work on media, it is valuable to know just how far dilution may be carried without encountering these difficulties. It is the hope of the authors that these researches may prove helpful to those who are dealing with the colorimetric determination of H-ion concentration in very dilute or weakly buffered solutions, and who will find it of vital importance to regulate the pH of the indicator solutions and that of the water used by controlling its CO_2 content.

8. The isohydric indicator method makes possible colorimetric salt error corrections and pH measurements to about 0.1 pH by the use of indicators (*a*) adjusted at their midpoint pH for testing buffer solutions of the usual concentrations and up to about 50 fold dilutions thereof; (*b*) adjusted at their lowest, midpoint and highest useful pH values for studying such buffers diluted about 50 to 1000 fold; and (*c*) adjusted at 0.1 or 0.2 pH intervals over their useful ranges for measuring highly diluted buffers or water. The correct pH point or range is shown by agreement between the pH as read and that of the indicator solution used. This method now gives us a new technique for measuring colorimetrically the pH values of very dilute buffer solutions or water, in which cases E.M.F. methods are either inaccurate or entirely useless.

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A STUDY OF TWO HUNDRED CULTURES OF GRAM-NEGATIVE BACILLI ISOLATED FROM CASES OF GENITO-URINARY INFECTION

J. H. HILL, L. R. SEIDMAN, A. M. S. STADNICHENKO AND M. G. ELLIS

From the James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Md.

We have hoped that a further study of the bacteria found in genito-urinary infections might be of value, both in increasing our knowledge of these organisms, and as a basis for later work. We present here the characteristics of two hundred cultures of Gram-negative bacilli, isolated from two hundred cases. As far as possible, these have been taken in their order of admission, but, in the four years' duration of this work, some cultures have been lost and a few have been discarded because of insufficient clinical data. Pseudomonas cultures, included in the preliminary report[1] have finally been omitted, in order to limit this series to organisms of the colon group, or related forms. Studies of the anaerobes and of the Gram-positive cocci found in genito-urinary infection are separate problems, about which almost nothing is yet known.

Our interest in studying bacillary infections has been first, to determine the bacteriological characteristics of the organisms, and, second, to consider any possible clinical correlation. The cultural characteristics of the organisms have been studied on a variety of media and the findings compared with those of other observers on bacteria from similar sources, or on related organisms. In a limited number of cases, it has been possible to study organisms present in the blood simultaneously with a genito-urinary infection, and a summary of these findings has been

[1] Paper read before the Society of American Bacteriologists, Philadelphia, December, 1926.

made.   In regard to any bacteriological-clinical correlation, comparisons have been made between the type of the culture and the nature of the infection.   There is reason to believe that a consideration of the wide differences of the strains might readily explain some of the marked variations in response to treatment of different "*B. coli*" infections, a point which is seldom made clinically.

PREVIOUS STUDIES OF GENITO-URINARY INFECTION

Although the literature on this subject is extensive, many of the articles are now of only historical interest, while in others, the emphasis is clinical rather than bacteriological, so that there are relatively few studies available for comparison.   We have prepared a bibliography of the more important general articles in which bacillary infections are discussed, and a few of these reports may be cited briefly.

Pasteur (1862) early stated his belief that the ammoniacal decomposition of urine was due to an organized ferment.   Later (1874) he is quoted as follows:

M. Pasteur, à l'occasion de l'intéressante note de MM. Gosselin et A. Robin, fait observer qu'il y aurait une grand utilité à rechercher si, dans tous les cas, ou dans des cas particuliers, la qualité ammoniacale de l'urine par la présence du carbonate d'ammoniaque n'est pas liée à l'existence d'un ferment organizé, notamment du ferment ammoniacal de l'urine si bien etudié par M. van Tieghem, ou de bactéridïes, ferments dont les germes seraient apportés de l'extérieur par le canal de l'urèthre, ou par le sang qui aurait pu lui-même prendre ce germe dans quelque partie du corps, par example, par une blessure quelconque, ou communication avec le canal intestinal; enfin ce germe, organisé vivant, peut être apporté souvent par une soude ou par un instrument chirurgical.

One may read many articles on this subject, which say less than has been expressed by Pasteur in these few words.

Early interest continued to center on Proteus infections. Roberts (1881) divided the bacillary infections into those with, and without, ammoniacal decomposition.   Attention was called to the polymorphic nature of the bacilli which did not liquefy

gelatin, to the pyogenic nature of infections with these organisms, and to the possibility of blood stream infection by Albarron and Hallé (1888). Doyen (1888) made an early study of renal infection. Guyon (1889) emphasized the rôle played by retention. The studies of Clado (1887a) (1887b) and of Krogius (1890) should be noted. Morelle (1891) included an examination of direct smears of specimens in his study of cystitis. The large number of infections due to non-gelatin-liquefying bacilli was emphasized by Krogius (1892), by Schmidt and Aschoff (1893), by Escherich (1894), by Savor (1894) and by Bastianelli (1895). The books of Melchior (1895) and of Rovsing (1897a) were important. Autointoxication was discussed by Goldburg (1895) and Hutinel (1896) also emphasized the probable intestinal origin of these infections.

Young (1898) made an interesting study of cystitis, cultures being obtained by suprapubic punctures, in order to avoid urethral contamination. Albarron, Hallé and Legrain (1898) summarized the literature. At the same time Rovsing (1897b) (1898) continued his studies, as did Melchior (1897) (1898).

Rostoski (1898) differentiated Colon group organisms and reported cystitides due to *Bact. acidi- lactici, Bact. lactis-aerogenes* and to *Bact. coli*. Maxwell and Clarke (1899) emphasized the secondary nature of *Bact. coli* infections, Suter (1901) studying them as secondary to tuberculosis. Parascandolo (1900) made both a clinical and experimental study. T. R. Brown (1902) provided bacteriological details in his careful report. Baisch (1903) studied post-operative cystitides in women and stated his belief that such catheter infections occurred only in pathological bladders. Von Hofmann (1904) made a thorough review of the literature. Raskai (1905) and Lenhartz (1907) differentiated their cultures bacteriologically.

Suter (1907) reported 211 cases of urinary infections, differentiating between those of endogenous and instrumental origin. Albeck (1907) studied the bacteriuria of pregnancy. Goldburg (1907) made a careful study of cystitis in infants, Jeffreys (1911) reporting 121 cases of urinary infection in children. Kodama and Krasnogorski (1913) found *Bact. coli* but seldom in extra-

renal infections and emphasized the necessity of considering kidney involvement when this organism was present. Tanaka (1909) studied 50 cases of cystitis, correlating the examination of smears with cultures, the latter being bacteriologically incomplete. Alsberg (1910) advised using the more general term "Colon Group" and noted morphological variations.

More recently, several valuable reports, such as those of Wolff (1912), of David (1914), of Brunnich (1918) and of Herrold and Culver (1919) are not available for purposes of comparison because no Voges-Proskauer tests were made. These authors, however, describe forms closely resembling those found by us. The high percentage of Colon group bacilli in renal infections has been emphasized by Campbell and Rhea (1918), by Mackenzie (1921) and by Mackenzie and Cochrane (1924a) (1924b). The work of Dudgeon (1908) and of Dudgeon, Wordley and Bawtree (1922) on *Bact. coli* infections of the urinary tract are of great importance, as are the studies of Kowitz (1915) and of Bitter and Grundel (1924). Murray, Williams and Wallace (1910), Beckman and van der Ries (1925) and others have demonstrated the immunological reaction of patients infected with *Bact. coli*.

METHODS OF STUDY AND FINDINGS

The routine laboratory methods used in the Brady Urological Institute have been described elsewhere by Young and Davis, (1926). They were closely followed in this series. We wish to acknowledge most gratefully the constant encouragement of Dr. Hugh H. Young and the clinical coöperation of Dr. Young and his associates in securing specimens with great care to prevent contamination. The method of collecting specimens consisted of cleansing the urinary meatus with alcohol and the anterior urethra by an injection of Meroxyl, 1:500. The first urine, in voided specimens, was allowed to escape and a specimen was then obtained in a sterile glass tube. The tubes used had been plugged with cotton and their tops wrapped in brown paper before sterilization. Many of the specimens were obtained by catheterization of bladder or kidneys. In the laboratory, rigid precautions were taken to avoid contamination. Specimens were

transferred for centrifugalization by means of sterile pipettes and were always kept in sterile tubes. Whenever the amount of the specimen allowed, it was centrifugalized at 2200 revolutions a minute for at least three minutes, often longer. This was sometimes possible even with prostatic secretions by using a finely pointed centrifuge tube.

We are convinced that there must be a careful correlation of thorough examinations of direct smears with cultural results, if accurate bacteriological findings are to be obtained from such specimens. Rarely, direct smears may fail to show organisms, which may be recovered regularly by culture, usually from cases undergoing intensive treatment. But, in general, an organism which develops but scantily on culture, usually only after forty-eight hours of incubation, and which has not been seen in the direct smear, can be discarded as a urethral contamination. Such contaminations, in the male, however, are almost invariably cocci. No culture has been included in which Gram-negative bacilli were not seen in the direct smear. One cannot, without special precautions, place any value on the estimation of the number of bacilli in such smears, because of the rapidity with which these organisms grow in urine. The recent report of Ver Mooten (1928) in which he reports that in over one third of his cases the examinations of direct smears and cultures do not correspond, is not in accord with our experience.

A Gram stain was done on all direct smears, a modification of Nicolle's (1895) one minute stain being used. This consisted of staining twenty seconds with gentian violet, (one part of saturated alcoholic gentian violet, freshly diluted with nine parts of 2.5 per cent aqueous solution of phenol), and then twenty seconds with Gram's iodin solution, decolorizing carefully with a mixture of one part of acetone in three parts of 95 per cent ethyl alcohol, washing in water and counterstaining twenty seconds with one part of carbol-fuchsin in twenty parts of distilled water.

MORPHOLOGICAL VARIATION

Although it is true that, in general, organisms of the genus Aerobacter differ from those of the genus Escherichia morpho-

logically, in being shorter and thicker and sometimes appearing in the form of streptobacilli or of diplococci, no presumptive diagnosis should be made from examinations of direct smears alone. Schmidt (1892) emphasized the diplococcoid forms which may be assumed by colon bacilli, a point also made by Adami, Abbott and Nicholson (1899) and by Konrich (1910). On the other hand, colon bacilli may assume equally confusing filamentous forms. The experimental work on morphological variation made by Péju and Rajat (1906), by Scales (1921) and by Henrici (1926) seems to correlate many of these variations with environmental changes. There is every reason to believe that such changes occur in different specimens of urine. Fishch (1926) has recently called attention to morphologic changes of *Bact. coli* in this medium. Clark and Ruehl (1919) have noted the difference in morphological characteristics between young and old cultures, which is of interest in view of the fact that many cases of genito-urinary infection have retention, the bladder serving as an excellent incubator for the *in vivo* growth of organisms.

Before sub-culturing, the organisms were plated by streaking lactose agar plates, and single colonies were fished to form the initial stock cultures. The fermentation of carbohydrates was studied on agar slants containing 1 per cent of the carbohydrate and the double indicators brom-cresol purple and cresol red (Manual of Methods for Pure Culture Study, 1926, A 22, B-25 and B-10). The agar was made sugar-free before the addition of the carbohydrate and care was taken not to overheat in sterilization. Inoculations were made both on the surface of the slant and deep into the butt. Observations were made for at least one week before negative cultures were discarded and tests were repeated whenever indicated.

For convenience our 200 cultures have been divided into 4 groups, as follows:

> Group I. 100 Escherichia cultures, fermenting lactose with acid and gas, not producing acetyl-methyl-carbinol, but methyl red positive.

Group II.   79 Aerobacter cultures, fermenting lactose with acid and
gas, producing acetyl-methyl-carbinol, but methyl red nega-
tive.

Group III.   5 Proteus cultures.

Group IV.   16 miscellaneous cultures.

The following carbohydrates were used:

    I. Monosaccharides:

        a. pentoses,

            1. arabinose, utilized by all forms in Groups I and II,
by none in Group III.

            2. xylose, utilized by all forms in Groups I and II,
and III.

            3. rhamnose, utilized by 96 per cent of Group I, by
all of Group II and none of Group III.

        b. hexoses,

            1. glucose, utilized by all of Groups I, II and III.

            2. galactose, utilized by all of Groups I, II and III.

            3. levulose, utilized by all of Groups I, II and III.

    II. Disaccharides:

        a. lactose, utilized by all of Groups I and II, by none of
Group III.

        b. maltose, utilized by all of Groups I and II, and III.

        c. sucrose, utilized by 55 per cent of Group I, and by all of
Groups II and III.

    III. Trisaccharides:

        a. raffinose, utilized by 51 per cent of Group I, by 96.2 per
cent of Group II, and, slowly, by 40 per cent of Group
III.

    IV. Polysaccharides:

        a. dextrin, utilized by 32 per cent of Group I, by 74.6 per
cent of Group II and 80 per cent of Group III.

        b. inulin, utilized by 6 per cent of Group I, by 15.1 per cent
of Group II and none of Group III.

        c. starch, utilized by 29 per cent of Group I, by 68.3 per
cent of Group II and 60 per cent of Group III.

    V. Glucosides:

        a. salicin, utilized by 31 per cent of Group I, by 97.4 per
cent of Group II and 40 per cent of Group III.

VI. Alcohols:

    a. trihydric alcohols.

        1. glycerol, utilized by all of Groups I, II and III.

    b. hexahydric alcohols,

        1. dulcitol, utilized by 69 per cent of Group I, by 63.2 per cent of Group II and 40 per cent of Group III.

        2. mannitol, utilized by all of Groups I and II and none of Group III.

        3. inositol, utilized by 8 per cent of Group I, by 87.3 per cent of Group II and by none of Group III.

    c. sorbitol, utilized by 80 per cent of Group I, by 93.6 per cent of Group II and by 10 per cent of Group III.

    d. adonitol, utilized by 13 per cent of Group I, by 83.5 per cent of Group II and by 10 per cent of Group III.

The value and correlations of these carbohydrate reactions will be discussed later in comparison with our findings.

THE PRODUCTION OF ACETYL-METHYL-CARBINOL

This was tested by adding 3 cc. of 10 per cent potassium hydroxide solution to 5 cc. of a five-day old broth culture. The medium used was a 1 per cent glucose, 1 per cent peptone and 0.5 per cent sodium chloride broth. After the addition of the hydroxide, the tubes were thoroughly shaken, the plugs removed and the cultures kept under observation for at least twenty-four hours. By making readings in comparison with negative controls, very satisfactory results were obtained. We preferred this method to allowing the color to appear only as a superficial ring, because the entire culture was colored and often remained so far several days, insuring a more satisfactory reading than could be obtained from the more transitory surface reaction.

Because it is an inconvenience to wait five days for this test, daily tests for five days were done with all of our Aerobacter cultures, inoculating the same lot of media with eighteen hour broth cultures of the organisms, using for inoculation a standard size of platinum loop, but with no other effort to equalize the inocula. The results of these tests are plotted in figure 1. By

this it will be seen that within twenty-four hours, forty-one cultures, or 51.8 per cent of this group showed definite production of acetyl-methyl-carbinol. There was little difference between the second and third day readings, sixty-eight cultures, or 86 per cent being positive the second day and sixty-nine cultures, or 87.3 per cent on the third. By the fourth day, seventy-one
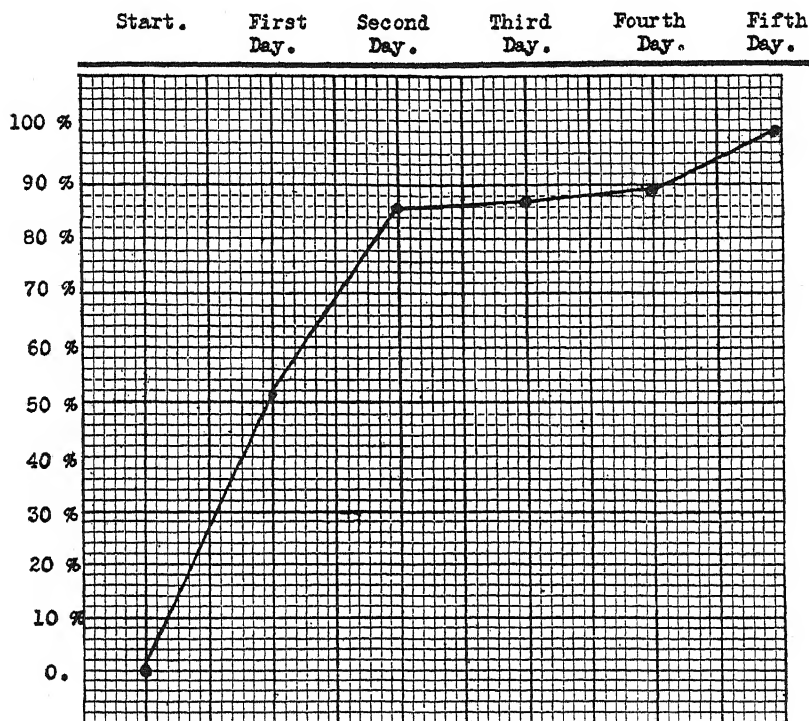
FIG. 1. PRODUCTION OF ACETYL-METHYL-CARBINOL BY SEVENTY-NINE CULTURES

cultures, or 89.8 per cent were positive and all were positive on the fifth day. Since 86 per cent were positive by the third day, we believe that it would be advisable to test part of a culture on the third day. This early production of acetyl-methyl-carbinol has been shown by Levine, Weldin and Johnson (1917).

The recent article by Paine (1927), in which the transitory nature of the Voges-Proskauer reaction is studied is of interest

here, since two of the three cultures which he found were positive from fourteen to thirty-six hours and then negative from three to ten days, were of renal origin. In our seventy-nine cultures, we have not observed this transitory action, but Paine's observation should certainly be noted in the study of atypical cultures.

In our series the Voges-Proskauer test has been satisfactory and has served as a valuable aid in identification. Correlated with citrate utilization, it might be of value in the classification of some of the encapsulated forms about which there remains so much confusion. There is evidence that at least some of the viscid, heavily encapsulated cultures do not utilize citrate and do not form acetyl-methyl-carbinol, while other cultures, similar in other reactions, utilize citrate rapidly and are Voges-Proskauer positive.

METHYL RED TEST

The medium used for this test was the glucose, potassium phosphate, peptone broth described in the A.P.H.A. Standard Methods of Water Analysis (1923). The addition of methyl red was carried out as there described, that is, by adding five drops of indicator (0.1 gram of methyl red in 300 cc. of alcohol, diluted to 500 cc. with distilled water) to 5 cc. of a five-day broth culture. Correlations of methyl red and of Voges-Proskauer tests in our Escherichia and Aerobacter groups were entirely satisfactory.

CITRATE UTILIZATION

This has proven to be one of the most valuable of all of the available tests. Three media were tried, Koser's (1924b) two citrate broths and Simmons' (1926) citrate agar. This last has now been adopted for routine use in preference to the broths and is invaluable in giving rapidly a presumptive generic classification, as citrate is utilized promptly by Aerobacter cultures and either not at all, or scantily and belatedly, by Escherichia forms. There are, of course, a few exceptions to this, which are found almost invariably in the types which are also intermediate in some of their other characteristics. One of our most typical Aerobacter strains, however, utilized citrate only slightly, al-

though repeated tests have been made with both freshly isolated and old stock cultures. The confusion which reigns at present in regard to the encapsulated forms makes it possible that further subdivision of these organisms will, in time, eliminate such inconsistencies. In general, the citrate test is so accurate, that one suspects the atypical nature of the culture rather than the test when results are contradictory. Paran (1925) has found the citrate test of value in tropical water analysis.

We have been interested in observing the different types of growth in citrate agar. Simmons (1926) found with the strains he studied that *Bact. coli* either failed to grow on citrate, or grew only as pin point colonies, the reaction of the medium being either unchanged or superficially acidified. Fifty-four of our one hundred Escherichia cultures failed to produce any visible growth on citrate agar in four days, with incubation at 37.5°C. Eighteen of the cultures produced only slight growth within four days, without visibly changing the reaction of the medium. Seventeen cultures definitely acidified the medium within four days, but only four of these grew well within two days. These four cultures belong to the Escherichia sub-group C, which seems to be nearest to the Aerobacter cultures. Eleven of the Escherichia cultures produced a deep blue coloration of the medium, this alkalinity developing in 4 cases only after forty-eight hours or longer. In the others,—all cultures of the intermediate subgroup,—it occurred promptly.

Although Simmons found that the *Bact. aerogenes* strains he tested uniformly produced an alkaline reaction on citrate agar, our seventy-nine Aerobacter cultures varied markedly in this respect. Thirty of our cultures or 38 per cent of the group, did produce prompt and permanent alkalinity. Fifteen cultures, or 19 per cent produced equally prompt and permanent acidity. Thirteen cultures, or 16 per cent, after an initial alkalinity, reverted to neutrality within seven days. Eight cultures, or 19 per cent, although growing promptly, were at first neutral and became alkaline only after forty-eight hours. Similarly, four cultures, or 5 per cent, growing promptly, remained neutral for forty-eight hours and then gradually became acid. Three cul-

tures, or 3.8 per cent, although growing well, remained neutral during seven days of observation. Two cultures, or 2.5 per cent, after an initial alkalinity, became acid within a week. Two other cultures, after an initial acidity, reverted to neutrality. One culture, after an initial acidity, became permanently alkaline and one culture utilized citrate only after forty-eight hours, the

TABLE 1

Citrate utilization by 100 cultures of the genus Escherichia and 79 cultures of the genus Aerobacter

	ESCHERICHIA		AEROBACTER	
	Number of cultures	Per cent	Number of cultures	Per cent
No growth....................................	54	54	0	0
Prompt growth with acidification................	4	4	15	19
Prompt growth with alkalinization..............	7	7	30	38
Prompt growth, medium remaining neutral......	0	0	3	3.8
Prompt growth with alkalinization, reverting to neutrality...................................	0	0	13	16
Prompt growth with delayed alkalinization......	0	0	8	10
Prompt growth with delayed acidification........	0	0	4	5
Prompt growth with alkalinization, reverting to acidity.......................................	0	0	2	2.5
Prompt growth with acidification, reverting to neutrality...................................	0	0	2	2.5
Prompt growth with acidification, reverting to alkalinity...................................	0	0	1	1.1
Growth only after 2 days incubation:				
Neutral.......................................	18	18	0	0
With acidification............................	13	13	0	0
With alkalinization...........................	4	4	1	1.1

growth even then being macroscopically invisible, but the medium becoming alkaline. No correlation of these variations could be made.

In our series, therefore, the value of the citrate medium has been more in the lack of, or in the rate of, growth than in the reaction changes of the medium. The difference between our Escherichia and Aerobacter strains was uniformly striking in

that the Escherichia strains either did not utilize citrate or did so only after forty-eight hours, while the Aerobacter strains in general utilized this medium promptly and heavily. On the other hand, both alkali and acid formers were found in both genera. These findings are summarized in table 1.

The stability of this citrate reaction, as shown by Koser (1924a) has been confirmed as most of the cultures have been tested both when originally isolated and after months of artificial cultivation.

LIQUEFACTION OF GELATIN

The medium was prepared as described in the Standard Methods of Water Analysis, A.P.H.A., (1923) page 95. After heavy inoculations, the cultures were placed in the incubator at 37.5°C. They were removed from the incubator at three or four day intervals, cooled on ice, and read in comparison with un-inoculated controls. Incubation was continued for at least two weeks before cultures were considered as not liquefying gelatin.

None of the Escherichia cultures liquefied gelatin, but twenty-one of the Aerobacter cultures and all of the Proteus forms did liquefy. Two of the latter lost this power after prolonged cultivation on artificial media. The gelatin liquefiers of the miscellaneous group will be discussed later.

PRODUCTION OF INDOL

Dunham's peptone broth, with Bacto-Difco peptone was used. The cultures were tested after two days of incubation, the Ehrlich method being used. (Manual of Methods, B. 31.) As excellent results were obtained in this way, neither tryptophane broth nor tryptic digestion of the peptone was tried. Cultures showing the characteristic pink color were shaken up with a little chloroform and not considered as indol positive unless the color was chloroform soluble. The fact that only eleven of our Escherichia cultures failed to form indol, while seventy-three, or 92.4 per cent of the Aerobacter series were indol negative is in accordance with the previous findings of others working with similar organisms.

MILK

After trying both litmus milk and milk containing brom-cresol purple, the former was selected as preferable. This was prepared by adding the indicator to cream-free milk, which was then tubed and autoclaved. Cultures in milk were observed for ten days. All of the Aerobacter cultures both acidified and coagulated milk. Ninety-six of the one hundred Escherichia cultures caused acidification and coagulation, three of them acidified without coagulation and one culture, No. 14, was alkaline. All of the Proteus cultures peptonized this medium. The action of the miscellaneous cultures on milk will be discussed later.

MOTILITY

This was studied by the examination of hanging drops of broth cultures. Tests were made with various ages of cultures, from eight to eighteen hours, and negative tests were always repeated. In the Escherichia series, twenty-nine of the one hundred cultures were motile. Of the Aerobacter cultures, twenty-one or 26.6 per cent were motile. Of these twenty-one cultures, however, sixteen belonged in the gelatin liquefying group and three of the remaining five showed only very slight motility. All five of the Proteus cultures were actively motile. De Stoecklin (1894), studying 300 undifferentiated strains of Colon group bacilli from faeces, found 116 motile forms and 184 non-motile.

UREA DECOMPOSITION

This was determined by the use of plain broth containing 2 per cent urea, phenolphthalein being used as an indicator and the medium adjusted to acidity. With the decomposition of urea and the production of alkalinity, there developed a pink color and a strong ammoniacal odor. Only one Escherichia culture decomposed urea, and this organism was in an intermediate group. Two cultures in the Aerobacter series, both of them gelatin liquefiers, decomposed urea. One of them has been isolated repeatedly from the same patient and always decomposes urea when freshly isolated, losing this power upon prolonged artificial cultivation. Unfortunately, we have been unable to follow the other culture

with repeated isolations. It has been carried for four years, however, and still decomposes urea promptly. All of the Proteus strains decompose urea energetically.

Pasteur's previously cited studies are of interest in this connection. Guiard's (1883) thesis was incomplete bacteriologically. Renault (1893) concluded that bacteria which do not liquefy gelatin do not decompose urea, a belief which cannot always be confirmed.

CAPSULE FORMATION

At the suggestion of Dr. J. H. Brown, the milk agar, India ink method was finally adopted for the demonstration of capsules. This was found to be much simpler than other methods and, if carefully prepared and controlled, entirely satisfactory. The medium consisted of one part of milk in nine of plain agar, only fresh, moist slants being used. By means of a platinum needle, a small amount of an eighteen-hour milk agar culture was transferred to a carefully cleaned glass slide, on which had been previously mixed one drop of sterile physiological salt solution and one drop of Higgins' American India Ink. The organisms were stirred gently but thoroughly into this large drop and a cover slip inverted over the mixture. Capsules could then be demonstrated beautifully by examining the preparation under the high dry magnification, manipulating the light and the fine adjustment screw simultaneously. The capsules stood out clearly as colorless halos around the bacteria and against the darker background of the ink. Negative results were checked by repeated examinations. The only difficulty with this method is that many of the freshly opened bottles of ink contain large encapsulated bacilli, such as were observed by Hamilton (1898) in similar inks. It is therefore necessary to run careful ink controls with every series of tests.

In view of the tendency of certain bacteriologists to place the encapsulated forms in a separate group, as in Castellani and Chalmers' (1919) "Tribe 10, Encapsulateae," of the Bacilliaceae, and Bergey's (1925) "Tribe Klebsielleae Trevisan, Genus Klebsiella." we have been interested in analyzing our cultures from this point of view. Our findings are summarized in table 2.

In our Group I, Escherichia cultures, fifty-five of the one hundred forms were not encapsulated, thirty-nine showed thin, but definite capsules, and six had thick capsules. These six cultures, however, all belonged to the intermediate sub-group D, which utilized citrate promptly. Four of these six cultures did not form indol and four of them were non-motile. The latter might be placed in Bergey's Genus Klebsiella.

In the Group II, Aerobacter, cultures, there was a marked increase in the number of encapsulated forms. Here only eight cultures or 10 per cent of the group were not encapsulated, while fifty-eight cultures, or 73 per cent showed small but definite capsules, and thirteen cultures, or 17 per cent were thickly encapsulated.

TABLE 2

Summary of capsule formation

	NO ENCAPSULATION		THIN CAPSULES		THICK CAPSULES	
	Number	Per cent	Number	Per cent	Number	Per cent
Group I, Escherichia..............	55	55	39	39	6	6
Group II, Aerobacter.............	8	10	58	73	13	17
Group III, Proteus...............	1	20	4	80	0	0
Group IV, Miscellaneous.........	1	6	6	37	9	56

Considering together these heavily encapsulated forms from both the Aerobacter and the Escherichia series, as would be necessary were they to be united in a genus of encapsulated forms, it is found that thick encapsulation is the only characteristic these organisms have in common, with the unimportant exception of inability to break down urea. Therefore, if such a genus or tribe is to be of value, it should be widened to include the forms described, of which one liquefied gelatin and four of which were motile. Taken as a whole, classification of the bacteria by their other characteristics, by which they fall easily into genera containing similar forms, would seem more satisfactory. Otherwise, we have widely differing organisms placed together in one group on the basis of a single characteristic. Moreover, if we include in a tribe of encapsulated organisms all of the forms which show any definite encapsulation, 45 per cent of the Escherichia cul-

tures and 90 per cent of the Aerobacter cultures would have to be included.   If there is to be any quantitative division on a basis of thick or thin encapsulation, by what standards is such a division to be made?

The relation of capsule formation to infection, studied by Preisz (1909) is further emphasized by Zinsser (1923). Reference should also be made to Furst's (1910) study and to the recent work of Avery, Heidelberger and Goebel (1925) on the isolation of a specific polysaccharide from a strain of Friedländer bacillus.

<center>HAEMOLYSIS</center>

Blood agar plates were prepared by adding two-thirds of a cubic centimeter of rabbit red blood cells, washed once with physiological salt solution to twelve cubic centimeters of agar. The plates were streaked with diluted twelve- or eighteen-hour cultures of the organisms and readings were made after incubation at 37.5°C. for twenty-four and forty-eight hours. Doubtful tests were repeated. We were surprised to find that sixty of the one hundred Escherichia cultures were haemolytic and also fifty-nine, or 74 per cent of the Aerobacter cultures. Only one of the Proteus cultures was haemolytic, although Kline (1925) has reported a haemolytic Proteus which was highly pathogenic for rabbits.

Table 3 shows the incidence of haemolytic organisms in different clinical conditions. It indicates, as was to be expected from the number of haemolytic cultures, that, in general, over 50 per cent of the organisms in any type of infection considered here are haemolytic.

Kayser (1903) in an interesting study of haemolytic colon group bacilli described a thermostabile colilysin, for which some normal sera contained an anticolilysin. Burk (1908) noted haemolytic colon group bacilli in both urine and faeces of suspected typhoid cases. Schmidt (1909) concluded that haemolysis was not correlated with fermentation reactions or virulence. Meyer and Lowenburg, (1924) expressed the opinion that the haemolytic colon bacilli could not be considered as one serologic group. Lowenberg (1924) found that haemolytic colon bacilli

played a rôle in pyelitis and were increased in the faeces in gastro-intestinal disturbances. Jinozzi (1925), on the other hand, agreed with Schmidt (1909) in considering the haemolytic proper-ties of colon group bacilli in the gastric tract unrelated to their virulence. Bawtree (1923) found thirteen haemolytic strains among thirty Escherichia cultures from urinary infections and emphasized their relation to the intestinal flora. Klingenstein

TABLE 3

Incidence of haemolytic organisms in various clinical conditions

CLINICAL CONDITION	TOTAL NUMBER OF CULTURES	HAEMOLYTIC CULTURES		NON-HAEMOLYTIC CULTURES	
		Number	Per cent	Number	Per cent
Renal infection..........................	57	38	67	19	33
Abscess formation......................	4	3	75	1	25
Blood stream invasion..................	12	7	58	5	42
Epididymitis...........................	23	13	56	10	43
Seminal vesiculitis.....................	11	7	64	4	36
Orchitis................................	1	1	(100)	0	0
Prostatitis.............................	60	41	68	19	32
Prostatic hypertrophy..................	62	40	64	22	35
Cancer of prostate.....................	7	5	71	2	28
Stricture, etc..........................	13	9	69	4	31
Cystitis, male..........................	115	82	71	33	29
Cystitis, female.......................	9	8	89	1	11
Urethritis.............................	10	6	60	4	40
Wound infections......................	11	9	82	2	18
Bacilluria, male.......................	6	3	50	3	50
Pneumaturia...........................	1	0	0	1	(100)
Arthritis..............................	7	5	71	2	28

(1925), studying forty-two cultures from the intestines, found that twenty-two were haemolytic. Of these, nine were from the jejunum, fourteen from the ileum and nineteen from the colon. Bitter and Grundel (1924) believed that haemolytic strains are found in acute cases, non-haemolytic in chronic cases. Dudgeon (1924) has described acute genito-urinary infections due to haemo-lytic bacilli which fermented lactose slowly.

Our four main groups of organisms will be considered separately before the general discussion.

As soon as any attempt was made to arrive at a specific identification of these organisms, difficulties were encountered. These will be discussed more fully under classification. We believe that it is more important to describe the characteristics of our cultures than to try to fit them into species which are either incompletely described or differently defined by several authors. (We have limited ourselves, therefore, to giving the closest specific identification.) It is impossible to publish the charts necessary to give full descriptions of all of our two hundred cultures,but the record of these is available for anyone who wishes to pursue the subject in greater detail than it is presented here.

Group 1. Escherichia

This group, consisting of one hundred cultures, or fifty per cent. of our series, was composed of cultures which fermented lactose with the production of both acid and gas, which failed to produce acetyl-methyl-carbinol, but which were methyl red positive. These cultures utilized citrate either scantily or not at all. Gelatin was not liquefied. Milk was usually both acidified and coagulated, but, in three instances, it was acidified without coagulation, and, in one case, it remained alkaline. The cultures varied in motility, encapsulation and haemolytic power. They regularly formed both acid and gas from arabinose, xylose, glucose, galactose, levulose, lactose, maltose, glycerol and mannitol. They usually fermented rhamnose and sorbitol. They varied in the fermentation of sucrose, raffinose, dextrin, starch, salicin and dulcitol. They seldom fermented inulin, adonitol or inositol.

These characteristics make these organisms conform in general to Winslow, Kligler and Rothberg's (1919) Group V., and to Bergey's (1925) or Weldin's (1927) Genus Escherichia. There were a few heavily encapsulated organisms which might have been placed in Castellani and Chalmers' (1919) Tribe Encapsulateae, or Bergey's (1925) Tribe Klebsielleae, perhaps wisely

omitted by Winslow, Kligler and Rothberg (1919) and, more recently, by Weldin (1927).

Summary of Group 1 cultures.   These 100 cultures fall into the following sub-groups.

  A. Typical sucrose negative cultures, 43, or 43 per cent of the group.
  B. Typical sucrose positive cultures, 40, or 40 per cent of the group.
  C. Atypical sucrose positive, citrate utilizers, 11, or 11 per cent of the group
  D. Atypical, heavily encapsulated, viscid cultures, 6, or 6 per cent of the group.

Niwa (1918–19) has reported a case of cystitis in which the infection was due to a bacillus which was sucrose negative, but which fermented raffinose, dulcitol and adonitol.   This organism is an interesting exception to the general correlation of sucrose and raffinose fermentation, to which we have only one exception. Niwa's organism is even more unusual in its fermentation of adonitol.   We had only one adonitol fermenter in our sucrose negative Escherichia cultures, our strain differing from Niwa's in being dulcitol and raffinose negative.   Riesman and Bergey (1908) have reported a case of pyelonephritis due to *Escherichia grünthali.*

GROUP II.   AEROBACTER

These cultures correspond to those described by Winslow, Kligler and Rothberg (1919) in their Group VI and by Bergey (1925) and Weldin (1927) in the genus Aerobacter.   They all formed acetyl-methyl-carbinol and were methyl red negative. They fermented carbohydrates more readily than the Escherichia cultures.   All of our seventy-nine Aerobacter cultures fermented sucrose and rhamnose as well as the carbohydrates utilized by all of Group I, namely, arabinose, xylose, glycerol, glycose, galactose, levulose, lactose, maltose and mannitol.   Many more of Group II than of Group I fermented sorbitol, raffinose, dextrin, salicin, starch, adonitol, inositol, and inulin, the difference being especially marked with sucrose, salicin, adonitol and inositol. On the other hand, more of Group I than Group II fermented

COMPARISON OF REACTIONS IN GROUPS I AND II
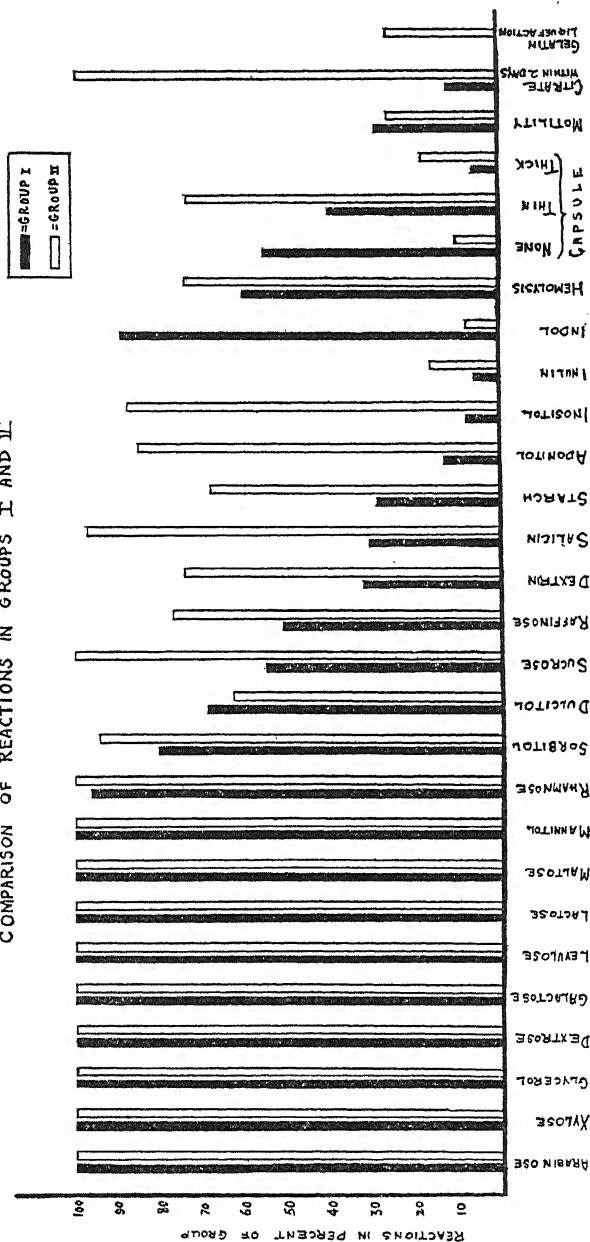
FIG. 2

dulcitol, this being the only carbohydrate showing this difference. The Group II forms were more generally indol negative than were the Group I cultures, an expected difference. More of the Group II cultures were haemolytic and many more were encapsulated and utilized citrate. Twenty-one Group II cultures liquified gelatin. These differences between the reactions of Group I and Group II are expressed in figure 2.

The Group II cultures have been divided into three sub-groups, as follows:

Sub-Group A, 21 gelatin-liquefying cultures.

Sub-Group B, 36 cultures, not liquefying gelatin, all fermenting all of the carbohydrates tested except dextrin, starch and inulin, on which the reactions varied.

Sub-Group C, 22 cultures, not liquefying gelatin, all fermenting all the carbohydrates tested, except adonitol, sorbitol, inositol, dextrin, starch and inulin, on which the reactions varied.

Sub-groups B and C might be considered together, having been separated as a matter of convenience, on account of the large number of cultures included.

Classification of Group II cultures. Although Grimbert and Legros (1900) and others have believed that the Friedländer group and the *Bact. aerogenes* group were identical, Coulter (1917) and others have emphasized the inability of true Friedländer group organisms to ferment lactose. Fitzgerald (1914) described the organisms of the Mucosus Capsulatus Group as non-motile, but some of them liquefied gelatin and 8 of his 43 cultures produced acetyl-methyl-carbinol. He could get no classification from a study of fermentation reactions. Small and Julianelle (1923) also found carbohydrate tests of little value in the classification of respiratory and granuloma strains of *B. mucosus-capsulatus*. Eight of their cultures, however, produced acetyl-methyl-carbinol, four of them being also methyl red positive. This interesting finding, we have not encountered in studying bacilli, but one of us (Stadnichenko) has found cocci which both produced acetyl-methyl-carbinol and were methyl red positive. We see no way, however, of differentiating non-motile, typical

Aerobacter cultures from the Voges-Proskauer positive forms of the *B. mucosus-capsulatus* group described by these authors.

The work of Levine (1916b) (1916c) (1917) (1921) and his colleagues is by far the most helpful contribution which has been made. The study by Levine and Linton (1924) of 123 strains of *Bact. aerogenes*, 76 from soil, and 47 from human dejecta, is very valuable. They confirmed the previously shown correlation between motility, gelatin liquefaction and glycerol fermentation. Their cultures were arranged in three groups. None of our gelatin liquefiers could be placed in their Group I, "Cloacae," because ours fermented glycerol. But our 21 gelatin liquefiers from urine fitted closely into Levine and Linton's (1924) intermediate group of cultures, which both liquefied gelatin and fermented glycerol. The only exceptions were that our cultures did not always ferment adonitol or inositol. We found adonitol of value in subdividing our gelatin liquefiers, as 12 of them utilized this alcohol and 9 did not. Our 58 Group II cultures which did not liquefy gelatin correspond very closely to the characteristics of Levine and Linton's (1924) Group II, or Aerogenes. Only four of these 58 cultures failed to ferment adonitol and would therefore be considered as "probably not of human origin." Two of these were thickly encapsulated and perhaps should be placed in another genus, should one be sufficiently defined. We also had two inositol negative cultures, but otherwise these organisms fit well into the classification of Levine and Linton (1924). The close correlation of our gelatin liquefiers with the intermediate group of Levine and Linton (1924) is confirmation of the value of their description of these organisms.

In addition to the articles cited in the general review of genito-urinary infections, a few may be noted which are of interest because the organisms described belong in these groups. Denys (1892) reported *Bact. aerogenes* in urine and tried to correlate its presence with similar findings in the faeces of children. Kidney infection, due to an encapsulated bacillus, was noted by Nicholaier (1894). Heyse (1894) reported pneumaturia due to *Bact. aerogenes*, only one case in our series being of this type. Trumpp (1896) described 29 cases of cystitis in children due to *Bact.*

aerogenes. Montt- Saavedro (1896) reported cystitides due to the Friedländer bacillus. Other reports have' been made by Barlow (1893), Warburg (1899), Wildbolz (1901), Wolf (1908) and Lenhartz (1906). Klieneberger (1908) discussed encapsulated bacilli as the cause of chronic cystitis. Luetscher (1911) gave an excellent review of the literature, but his belief that the percentage of *Bact. aerogenes* infections had been overestimated now seems based on incomplete bacteriological differentiation. The work of Meyer and Hinman (1920) and of Lacy and Murdoch (1922) on lactose negative, encapsulated bacilli is related more closely to the encapsulated forms we have described in our Group IV.

GROUP III.   PROTEUS

There were only five cultures in this group. They all fermented xylose, glucose, sucrose, maltose and glycerol. None fermented arabinose, rhamnose, lactose, inulin, mannitol or inositol. They varied in the fermentation of dulcitol, salicin, sorbitol, adonitol, raffinose, dextrin and starch. Although van Loghem and van Loghem-Pouw (1912), Bertholet (1913) and Wenner and Rettger (1919) have found that their Proteus strains generally formed indol, and Steensma (1906) has observed a pseudo-indol reaction, none of our strains formed indol by the method used. This agrees with the findings of Groot (1918), of Besson and Ehringer (1923) and of van Loghem (1918). None of our strains formed acetyl-methyl-carbinol, although Archibald (1913) has described a Proteus form which did. All of our cultures were methyl red negative.

After an initial acidification, these cultures showed an alkaline reaction in milk, with partial or complete peptonization. Gelatin was always liquefied by freshly isolated cultures. Urea was always decomposed and much more actively than in any other group. The utilization of citrate agar varied, all cultures showing but scant growth on this medium, four giving an alkaline reaction and one remaining neutral. All cultures were actively motile. Only one was haemolytic.

According to both Bergey (1925) and Weldin (1927), all of

these cultures were *Proteus vulgaris*, differentiated on the basis of their action on maltose and mannitol.   Our forms differed, however, from the specific definition of Weldin (1927) in that they were indol negative and sometimes fermented dextrin.

The carbohydrate reactions of this group were in some cases much less clear-cut than in Groups I and II and gas production by these Proteus strains was somewhat irregular and in no case marked.   For example, in sucrose, although acid was produced by all, in some cases gas was not evident until from the third to the fifth day, and in one case no gas was noted.   In both maltose and glycerol, although acidification was produced by all cultures, gas production was variable.

The loss of ability to liquefy gelatin is in accordance with the findings of Theobald Smith (1894), of Herter and Ten Broeck (1911) and others.   Three of our Proteus cultures have retained their power to liquefy gelatin, while two have lost it entirely.

No pigment production was exhibited by any of our strains. They were cultured for fourteen days on plain broth by the method of Bengtson (1919) and on potato for five days as suggested by Jordan (1903), but failed to show pigment by either method.

Only one of the Proteus cultures was haemolytic.   This differed from the haemolytic strain described by Kline (1925) in that ours did not ferment mannitol or arabinose, and did ferment xylose.   There were also some differences in gas production.

Ability to grow under anaerobic conditions was tested on chopped meat medium, incubated at 37.5°C. for fourteen days. Growth was obtained in all cases.   The meat became slate-colored and there was a slight reduction in the size of the meat particles.   No gas was produced and in no case was there evidence of proteolytic action.

Many authors have reported urological infection due to Proteus.   Lenhartz (1923) found it causing septicaemia of renal origin.   Wolff (1912) found that 8 per cent of his one hundred cultures were Proteus.   He divided these eight cultures into 5 types, only one of which fermented mannitol.   The recent work of Hagar and Magrath (1925) (1926) (1928) is of special interest.

The organism which they find as the etiological factor in encrusted cystitides they now call *Proteus ammoniae,* as it seems more closely related to this genus than to Salmonella, although sucrose negative. We have not found this organism in our series, but the increase in the incidence of Proteus cultures in lithiasis, as shown in the clinical summary, and the promptness with which they all decompose urea makes them of importance in alkaline infections. The problem is complicated by the fact that such infections are almost always mixed, although Hagar and Magrath have obtained pure cultures from some of their cases.

Group IV.   Sixteen miscellaneous cultures

These may be placed in the following genera:

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Alcaligenes.............................................1 culture
Eberthella.............................................5 cultures
Shigella...............................................9 cultures
Salmonella.............................................1 culture
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Alcaligenes.   This culture, motile and gelatin liquefying, may be considered as *Alcaligenes bookeri,* Bergey (1925, page 258), or Weldin (1927, page 187). It was associated with a coccus in a case of pyonephrosis and epididymitis. Beckmann and van der Reis (1925) have described cystitis due to this organism.   Straub and Krais (1914) and others have found it in urinary infections, Mackenzie and Cochrane (1924b) reporting it in 63 of their 241 ureteral cultures, an unusually high incidence.

Eberthella.   Using the classification of Bergey (1925), both motile and non-motile organisms which ferment glucose without gas belong in this genus, while Weldin (1927) places the non-motile forms in Shigella.   Fourteen of our Group IV cultures may be placed in these genera, seven cultures not fermenting lactose and seven fermenting lactose without gas.

Four of our cultures were practically identical.   They were motile, liquefied gelatin, and in other ways resembled Ford's (1901) *Eberthella chylogena* except for their action in milk, and resembled his *Eberthella dubia,* except for their action in milk and their failure to form indol. These organisms differed from *Eberthella lewisii* in the liquefaction of gelatin, Weldin (1927, page

172). They seem, therefore, more closely related to the organism described by Ford (1901).

One motile culture was considered as *Eberthella talavensis*. One non-motile culture could not be classified. It resembled *Shigella dysenteriae* closely and, although differing in its fermentation of mannitol from *Shigella paradysenteriae* Flexner, it showed an immunological relationship to this organism, agglutinating in its serum in a dilution of 1:40. Another culture, non-motile and lactose negative, was apparently *Eberthella alkalescens*, or *Shigella alkalescens*.

The remaining seven cultures in Group IV, which all fermented both glucose and lactose without gas, as well as mannitol and xylose, were non-motile. The four dulcitol negative forms were considered as *Shigella madampensis*, Weldin (1927, page 181). The dulcitol positive forms seemed to be *Shigella ceylonensis*, Weldin (1927, page 182). They satisfied the definitions of this species much more closely than that of *Eberthella viscosa*, the closest resemblance to be found in any of the forms described by Bergey (1925, page 254).

One culture has been considered *Salmonella paratyphi*, in spite of its fermentation of sucrose, because it was agglutinated by *Salmonella paratyphi* immune serum in a dilution of 1:320.

Dudgeon (1906) has described an organism from a case of prostatitis which closely resembled some of our Group IV cultures, although we had no salicin positive, sucrose negative forms and his organism was slightly motile. Lacy and Murdoch (1922) have reported encapsulated non-gas-forming bacilli from the urinary tract. One of our cultures resembles the organism described in its fermentative reactions. Meyer and Hinman (1920) have described a similar encapsulated organism from a case of hydronephrosis.

BACTERIOLOGICAL SUMMARY AND DISCUSSION

The 200 hundred cultures fall into 65 types, if grouped according to their 14 most clearly differentiating characteristics, that is, their behavior in regard to the Voges-Proskauer test, methyl red, citrate, gelatin, milk, indol, encapsulation, motility

and the fermentation of glucose, lactose, sucrose, dulcitol, salicin and adonitol.   Our 100 Escherichia cultures form 30 of these 65 types, while the 79 Aerobacter cultures form 20 types.   The largest number of cultures in any Escherichia type was 15, while the largest number in any Aerobacter type was 29.   That is, the Aerobacter cultures tended to be less differentiated.   The 5 Proteus cultures formed 3 types, differing only in their fermentations of dulcitol, salicin and adonitol.   The 12 types of the miscellaneous cultures are not significant.

While these 65 types represent only a few of the number theoretically possible on the basis of 14 characteristics, there seems no reason why many others should not be found in addition to those previously reported from other sources.   The use of some outline which includes the possible variations of whatever characteristics may be considered essential, and which gives each variation a place, would seem simpler than the available systems of classification.   Levine (1918), considering the possibilities presented by Bergey and Deehan (1908) and by others too flexible, on account of the large number of possible variations, has emphasized the necessity of studying correlations of characteristics.   Such a statistical plan would offer some way of quickly making comparisons and of placing organisms not previously listed, while the demands of nomenclature could be satisfied by naming these types as found.

In regard to classification, a review of the enormous literature on the subject has yielded comparatively few studies that are helpful in the light, or rather darkness, of present knowledge.   Moreover, these few disagree in so many ways or have such omissions, that the unfortunate bacteriologist who tries to identify cultures by them, is at once thrown into confusion.   There are, however, some valuable guides, which will be discussed briefly.

Winslow, Kligler and Rothberg (1919), basing their study on 160 cultures of the colon-typhoid group, offer a grouping which is valuable in the very breadth of its classification, which allows it to be used many times when the more detailed definitions of others exclude cultures on minor characteristics.   However, they frankly offer no assistance in regard to encapsulation.   They

have no place for anaerogenic lactose fermenters.   Their Group
V, in which most of our Group I, Escherichia, cultures belong, has
no place for motile species which ferment both salicin and sucrose.
This omission excludes nine of our cultures.   In their Group
VI, in which most of our Group II, Aerobacter, cultures belong,
the species *B. cloacae* is defined as non-motile, so excluding six-
teen of our twenty-one gelatin liquefying cultures.   By the ad-
dition of such groups to their classification, the fundamental
groupings of Winslow, Kligler and Rothberg (1919) could
be made to include these and other forms which may be found.

Castellani and Chalmers (1919), emphasizing gelatin liquefac-
tion and encapsulation in tribal differentiation, would force us to
remove the 45 encapsulated cultures from our Group I, Escheri-
chia.   While the advisability of taking away from this group the
6 heavily encapsulated, viscid cultures may be seriously con-
sidered, it seems illogical to remove the 39 thinly encapsulated
cultures from forms which they so closely resemble in every other
way.   In their Tribe Encapsulateae, Castellani and Chalmers
(1919) describe only two species which ferment both glucose and
lactose with acid and gas.   Of these, *Encapsulatus acidi-lactici*
is Voges-Proskauer negative, and does not ferment inositol.
Seven of our encapsulated Group I cultures did ferment this
alcohol, although three of these were thickly encapsulated and
most fitted to be placed in this tribe.   The only other glucose
and lactose fermenting species of the Tribe Encapsulateae, or
Encapsulatus lactis-aerogenes, is described as Voges-Proskauer
positive, and would, therefore, include all of the encapsulated
forms in our Group II which did not liquefy gelatin.   Further
division of this tribe would be useful.   The gelatin-liquefiers,
according to Castellani and Chalmers (1919) would be placed in
the Tribe Proteae, Genus Cloaca, species cloacae.   This is a diffi-
cult transition, in view of the fact that these organisms seem more
closely related to the other Voges-Proskauer positive, lactose fer-
menting, milk coagulating cultures, than to the Voges-Proskauer
negative, lactose negative, milk peptonizing Proteus cultures.
Castellani and Chalmers (1919) are more helpful than others in
the identification of atypical or unusual forms.

Bergey's (1925) Manual is of value in its genera of the tribe Bacterieae, but confuses by such mistakes as dividing the genus Escherichia on the fermentation of lactose and glucose, (page 217, AA. 1 and AA.1a bb c), when the key describes the genus as forming both acid and gas in these carbohydrates.   The problem of encapsulation offers much of the confusion found in Castellani and Chalmers (1919).   The non-motile, encapsulated organisms are placed in the genus Klebsiella, but no way is given of clearly differentiating the glucose and lactose fermenters in this genus from Aerobacter cultures, as there is no statement as to the production of acetyl-methyl-carbinol by the Klebsielleae.   There is no place for motile encapsulated organisms in this tribe, or any way to distinguish Voges-Proskauer negative, encapsulated, lactose fermenters of this tribe from certain Escherichia forms.   It it seldom possible to make more than a generic classification by Bergey's (1925) system, but his genera are of value.

Weldin (1927) is more helpful than Bergey (1925) in many ways, but again throws one into confusion by putting the encapsulated forms under Proteus.   This is inconsistent if any encapsulated lactose fermenters are to be included.   Weldin (1927) offers no species of the Genus Escherichia which differs in the fermentation of dulcitol and salicin.   Our 17 cultures which did differ in the fermentation of these carbohydrates form such a large and consistent group that we believe such a subdivision should be made.   Weldin (1927) omits the use of salicin in his definition of *Escherichia communior*, defined as not fermenting this carbohydrate by Winslow, Kligler and Rothberg (1919) and as salicin positive by Bergey (1925).   This is a typical example of the perplexities which confront anyone who endeavors to use the present methods of classification.   Weldin (1927), however, remains the most useful at present for specific identification.

COMPARISON WITH RELATED FORMS FROM INTESTINAL CONTENTS

In view of the many studies which have been made of Colon group bacilli from other sources, certain comparisons may be made.   The findings of others in studying faecal strains of the Colon group show a relatively high percentage of *Bact. coli*, or

Escherichia cultures, in proportion to the percentage of *Bact. aerogenes* or *Bact. cloacae* (Aerobacter) strains. These findings have been summarized in table 4.

From this it will be seen that, in the fourteen reports tabulated, representing nearly 7000 cultures, some authors found no Aerobacter strains, others from 0.06 to 16 per cent. Rogers, Clark and Lubs (1918), using special enrichment methods, obtained 26 per cent Aerobacter cultures in faeces. These results are in striking contrast to the presence of Aerobacter strains in 39.5 per cent

TABLE 4

Colon bacilli in faeces

AUTHOR	TOTAL NUMBER OF CULTURES	BACT. COLI		BACT. AEROGENES AND BACT. CLOACAE	
		Number	Per cent	Number	Per cent
Chen and Rettger (1920)...............	173	173	100	0	0
Rogers, Clark and Evans (1914)........	150	149	99.9	1	0.06
Levine (1916a).........................	117			0	0
Koser (1924)..........................	118	109	92.4	9	7.6
Schöbl and Ramirez (1925).............	20	20	100	0	0
Rogers, Clark and Lubs, (1918)........	177	131	74	46	26
MacConkey (1909).....................	316	306	96.6	11	3.4
Darling (1919)........................	113	113	100	0	0
Stokes, (1919)........................	156	131	83.9	25	16
de Magalhaes (1924)...................	1,290		96.5		3.1
Robinson (1920).......................	2,100		93.4		6.1
Clemesha (1912).......................	2,236			184	8.0
Hulton (1916).........................	13	13	100	0	0

of the urological infections, or in 44.1 per cent of the 179 true Colon group infections in our series.

If the urological infections are of intestinal origin, as has been believed by many, including Heitz-Boyer (1919), Bawtree (1923), and Chynoweth (1926), it is possible that the Aerobacter strains respond to some selective action in the genito-urinary tract. More probably, these forms, being more resistant than Escherichia strains, survive when introduced into the genito-urinary tract, while the Escherichia organisms, although far outnumbering the Aerobacter forms in faeces, are more easily killed when trans-

planted.   This same point may be made in regard to Colon group invasions of the blood stream, as will be shown later.   The problem of whether or not there is any increase in the Aerobacter content of the intestines before or during genito-urinary infection, is one about which we have no information.

CLINICAL SUMMARY

By comparing the incidence of our four main groups of cultures to their proportion in the whole series of 200 cultures, some estimate may be made of variation in different clinical conditions. We may state briefly the few correlations which seem important.

1. Renal infections, 57 cases, 49, or 86 per cent pure cultures. The only variation to be noted here is the increase in incidence of gelatin liquefying forms of the genus Aerobacter.   Of the 21 Aerobacter strains isolated from renal infections, 9, or 42.8 per cent liquefied gelatin, although only 26.5 per cent of all of the 79 Aerobacter cultures did so.   The fact that in 8 of the 9 cases of renal infection due to gelatin-liquefying bacilli, the organisms were obtained in pure culture, seems to offer evidence that these strains, although commonly considered harmless, are not lacking in pathogenicity when established in the kidney.

2. Urolithiasis, 25 cases, 17, or 68 per cent pure cultures.   Here there was a sharp drop in the incidence of the Escherichia cultures to 28 per cent, less than half of these being pure cultures. There was a corresponding increase in the incidence of cultures from Group II, Aerobacter, and Group III, Proteus.   The Aerobacter cultures had an incidence of 48 per cent in lithiasis, 83.3 per cent of them being pure.   The Proteus cultures, forming only 2.5 per cent of the total of 200 cultures, occurred in 12 per cent of the cases with stone formation.

3. Abscess formation, 4 cases, 3, or 75 per cent pure cultures. One prostatic abscess, due to a pure Escherichia strain was encountered.   The other three were cases of peri-urethral abscesses. Two of these three cases had blood stream invasions, a pure culture of a Shigella strain being obtained from both blood and abscess in one instance and a pure culture of a gelatin-liquefying Aerobacter strain being obtained from the blood in the second

case, although associated with a Gram positive coccus in the abscess. A pure, non-gelatin-liquefying Aerobacter strain was obtained from the third case of peri-urethral abscess.

4. Epididymitis, 23 cases, 19, or 82.8 per cent pure cultures. All of the 11 Escherichia cultures obtained from cases of epididymitis were pure. There was a relative increase of sucrose negative Escherichia strains. No gelatin liquefying bacilli were found among the 9 Aerobacter strains encountered. There was 1 pure Proteus culture. One Alcaligenes, mixed with a coccus, was found, and 1 pure culture of a Shigella strain.

5. Seminal vesiculitis, 11 cases, 10, or 90.9 per cent pure cultures. The incidence of Escherichia cultures here was above normal, being 72.7 per cent. The percentage of heavily encapsulated Group I, Escherichia, cultures was increased, being 37.5 per cent as compared with only 6 per cent in the total Group I series. There was a drop in the incidence of Group II, Aerobacter, cultures, to 27 per cent, all pure cultures. No gelatin liquefiers were found, but two of the Aerobacter cultures were heavily encapsulated, bringing the number of heavily encapsulated cultures found in seminal vesiculitis to 5 or 45.4 per cent.

6. Prostatitis, 60 cases, 48, or 80 per cent pure cultures. There was no specific incidence in these infections, nearly every type of organism being found. The studies of Young, Geraghty and Stevens (1906), of Dudgeon (1906), (1907), of Culver (1916), of Rosen (1919), and of Baker (1925) may be cited here, although bacteriologically incomplete.

7. Prostatic hypertrophy, or carcinoma of prostate with retention, 69 cases, 45, or 65.2 per cent pure cultures. There was no specific incidence. The relatively small number of pure cultures was to be expected.

8. Stricture, contracture of the vesical orifice, with retention, 13 cases, 12 or 92.3 per cent pure cultures. There was a slight increase in the number of Aerobacter cultures, to form 53.9 per cent of the group.

9. Cystitis, male, 115 cases, 85, or 73 per cent pure cultures. Some difficulty was encountered in deciding what to include in this group. Using clinical diagnoses, and cystoscopic reports,

but omitting doubtful cases, some of which perhaps should have been included, these 115 cases may be considered as representative. There was no significant variation in incidence of culture groups. While 83.8 per cent of the 62 Escherichia cultures were pure, only 38.2 per cent of the 43 Aerobacter cultures were pure, a proportion which by no means held in other clinical types. There were 2 Proteus infections, 1 pure and 1 mixed. There were 4 Eberthella infections, only 1 pure, and 4 Shigella, all pure.

10. Cystitis, female, 9 cases, 8, or 88.8 per cent pure cultures. There was a relative increase of Aerobacter cultures, to form 55.5 per cent of the infections.

11. Urethritis, 10 cases, all pure cultures. Ninety per cent of the cultures were Escherichia.

12. Bacilluria, male, 6 cases, 5, or 83.3 per cent pure cultures. Escherichia cultures formed half of these infections.

13. Wound infections, 11 cases, no pure cultures. No Escherichia cultures were found in wound infections, 81.8 per cent being Aerobacter. There was 1 Proteus infection and 1 Shigella.

14. Arthritis, 7 cases, 6, or 85.7 per cent pure cultures. Four of these were Escherichia, all pure, and 3 Aerobacter, 2 pure. Kauntze (1924) has recently discussed the rôle of *Bact. coli* in arthritis.

15. Blood stream invasions, 13 cases, 12 of bacillary infection, all pure cultures. These thirteen cases had blood stream invasions simultaneously with genito-urinary infection. In one of these, *Staphylococcus aureus* was recovered from the blood, but not from the urine. The other twelve cases showed the same bacillus in the blood which was also found in the genito-urinary tract. Of these twelve cultures, only one, or 8.3 per cent was from Group I, Escherichia. That is, there was a great drop in the incidence of this group, which was normally 50 per cent. On the other hand, nine of the twelve bacillary blood invasions, or 75 per cent were by organisms of Group II, Aerobacter. There was no blood stream invasion by Proteus in our series and only two, or 16.6 per cent from Group IV organisms. One of these was an Eberthella and the other a Shigella. It is evident, therefore, that, just as in general there is a marked increase of the in-

cidence of Aerobacter cultures in genito-urinary infections in proportion to the known incidence of these organisms in the body, there is a similar strikingly high percentage of these Aerobacter species in blood stream invasions. This is a small series of cases, but further evidence is gradually being accumulated which supports this finding. Its significance cannot be explained unless on the grounds previously discussed.

A review of the many articles on Colon group invasions of the blood stream fails to reveal any previous attempt to identify such cultures generically, except in a few separate cases. The importance of the genito-urinary tract as the focus for such invasions, however, has long been emphasized, especially by Felty and Keefer (1924), by Jacob (1909) and by Maciag and Olbrycht (1926). Liebermeister (1906) cites Barlow's early case of *Bact. coli* sepsis and death in a case of urethral stricture. Krencker (1907), Helmoltz (1926), Rooke (1925) and Roux and Lemaire (1925), among others, have reported *Bact. coli* septicaemias of urinary origin, without further bacteriological differentiation. Friedländer bacillus septicaemias have been reported by Brouardel (1926), by Lereboullet and Pierrot (1927), by Caussade, Joltrain and Surmont (1924) and others. Berg and Libman (1902), Longcope (1902), and others, have reported paracolon septicaemias.

FINAL SUMMARY

1. Two hundred cultures of the Colon group, or related organisms, isolated from two hundred cases of genito-urinary infection, have been studied.

2. These cultures may be divided as follows:

Group I. One hundred cultures, or 50 per cent of the series, were methyl red positive, Voges-Proskauer negative, and may be considered as *Bact. coli*, or closely related forms. They belong in Winslow, Kligler and Rothberg's Group V, or in Bergey or Weldin's Genus Escherichia. They may be subdivided into 4 subgroups, namely: (A) 43 typical sucrose negative cultures; (B) 40 typical sucrose positive cultures; (C) 11 typical sucrose positive cultures which utilized citrate promptly; (D) 6 atypical,

heavily encapsulated cultures, which might have been placed in another genus.

Group II.   Seventy-nine cultures, or 39.5 per cent of the series, were methyl red negative, Voges-Proskauer positive, and may be considered as *Bact. cloacae, Bact. aerogenes*, or closely related forms.   They correspond to Winslow, Kligler and Rothberg's Group VI, and to Bergey's or Weldin's Genus Aerobacter.   They may be subdivided on the bases of gelatin liquefaction into two groups, as follows: (A) 21 gelatin liquefying cultures, corresponding closely to Levine and Linton's intermediate group; (B) 58 cultures, which did not liquefy gelatin, and which resemble closely the organisms described by Levine and Linton in their Group II.

Group III.   5 cultures, or 2.5 per cent of the series were Proteus.

Group IV.   16 cultures, or 8 per cent of the series composed our miscellaneous group, which may be subdivided as follows:

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Alcaligenes,...............................................1 culture
Eberthella.................................................5 cultures
Shigella...................................................9 cultures
Salmonella.................................................1 culture
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3. Encapsulation.   135 cultures, or 67.5 per cent of the series showed definite encapsulation.   Of these, 28 had thick capsules. A comparison of the encapsulated forms, however, showed that, if they were placed in a separate genus, it would have to include species varying in every other characteristic but encapsulation. We do not believe, therefore, that until more is known about encapsulation, it is a satisfactory basis for generic differentiation.

4. Citrate utilization.   This has been found to be a very valuable means of differentiation, Simmons' citrate agar being more useful than Koser's citrate broths.   Citrate was used scantily or not at all by our Group I (Escherichia) cultures, with the exception of 11 strains which were also intermediate in their other characteristics.   Citrate was used rapidly by all but 1 of our Group II (Aerobacter) cultures, and by all of our Proteus cultures.   It was not utilized by our one Alkaligenes culture, or

our one *Salmonella paratyphi* culture.   The correlation between citrate utilization and the Voges-Proskauer test was excellent. The citrate test is of great value in obtaining within twenty-four hours a presumptive generic differentiation between Escherichia and Aerobacter.

5. *Voges-Proskauer test.*   It was found by testing our 79 Aerobacter cultures daily for five days, that 51.8 per cent were positive within twenty-four hours, 86 per cent were positive in two days, 87.3 per cent in three days, 89.9 per cent in four days and 100 per cent in five days.   It is therefore evident that in about 86 per cent of such cultures, testing for the production of acetyl-methyl-carbinol on the third day would give a positive result, saving 2 days time in making generic identification.   Cultures negative on the third day could be held until the fifth day, as is customary.

Haemolysis.   The number of haemolytic cultures was higher than had been anticipated, 131 cultures, or 65.5 per cent of the series being haemolytic.   In group I (Escherichia,) 60 per cent of the cultures were haemolytic, in Group II (Aerobacter) 74 per cent.   In Group III (Proteus) only 1 of the 5 cultures was haemolytic.   In Group IV, the haemolytic cultures included the one Alcaligenes found, 2 of the 5 Eberthella cultures, 7 of the nine Shigella cultures and the one Salmonella culture.

A comparison of haemolytic action with clinical types of infection shows a high percentage of haemolytic cultures in many types of infection, but with no significant selective pathogenicity on the part of these strains.

Urea decomposition.   This was found most markedly in the Proteus cultures, 2 of the Group II (Aerobacter) strains also possessing this power although their action on urea was much weaker than that of the Proteus cultures.   Although the Proteus strains did not lose their ability to decompose urea after artificial cultivation, one of the Aerogenes strains could decompose urea only when freshly isolated from urine.

Comparison with intestinal Colon group cultures.   A review of the literature on colon group strains from intestinal contents, revealed the fact that in urological infections the incidence of

Aerobacter cultures is much higher than in the intestines. The reason for this is unknown.

Clinical summary. The most significant facts found in comparing our culture groups with different clinical infections were as follows:

1. Seventy-five per cent of the blood stream invasions were due to organisms of Group II (Aerobacter), in the 12 cases in which the same organism was recovered from blood and urine, the incidence of Group I (Escherichia) cultures being only 1 case, or 8.3 per cent. This high proportion of Aerobacter cultures in such blood stream invasions parallels the high incidence of this group of cultures in genito-urinary infections, as compared with organisms present in the intestinal flora. The incidence of Aerobacter cultures in the blood, however, is even higher than in the urine. So far as we have been able to determine, this differentiation of the Colon group in blood stream invasions has not been made previously and further data should be obtained.

2. There was also a drop in the incidence of Group I (Escherichia) cultures in the 25 cases of lithiasis, with a corresponding increase of Group II (Aerobacter) cultures to 48 per cent and of Proteus to 12 per cent.

3. Two of the three cases of abscess formation developed blood-stream invasions.

10. We hope that by this analysis of 200 cultures, some knowledge of the nature of the bacillary infections has been obtained which will be of value as a basis of further comparison and a point of departure for further study.

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EFFECT OF TEMPERATURE ON BACTERIAL NUMBERS IN DIGESTING SEWAGE SLUDGE[1]

H. HEUKELEKIAN[2] AND WILLEM RUDOLFS[3]

Agricultural Experiment Station, New Brunswick, New Jersey

In recent years attention has been called to the influence of temperature on sewage sludge digestion. It has been pointed out that in the winter months the temperature prevailing in the digestion tanks in northern climates is too low for efficient digestion and that great benefits are to be derived from heating the tanks. A temperature of 21°C. (70°F.) has been found to be the most economical and practical limit; however, higher temperatures accelerate the rate of digestion considerably. The optimum temperature lies between 27° and 30°C.; temperatures beyond this have a retarding effect.

Little has been known about the bacteriological changes underlying the differences in the rate of digestion at different temperatures, although we know that activities of the organisms are accelerated with increased temperatures (within limits). The total numbers may or may not change. Presumably, certain groups of bacteria are stimulated but their presence in the total plate counts may be over-shadowed.

The following paper is an attempt to point out some of the bacteriological differences occurring during decomposition of solids at different temperatures.

METHODS

The fresh solids were collected from the Plainfield sewage disposal plant and brought to the laboratory. They were divided

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[1] Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Sewage Disposal.

[2] Research Bacteriologist.

[3] Chief, Department of Sewage Disposal.

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into portions, placed into different bottles and incubated at 10°, 18°, 24°, 29.5° and 35°C. A duplicate series was incubated at the same temperatures with lime added to correct the reaction. This report deals only with the bacteriological results obtained from the experiment, which included in addition a complete series of chemical and protozoological studies.

The bacteriological analyses consisted of total plate counts and estimates of certain general groups of bacteria such as
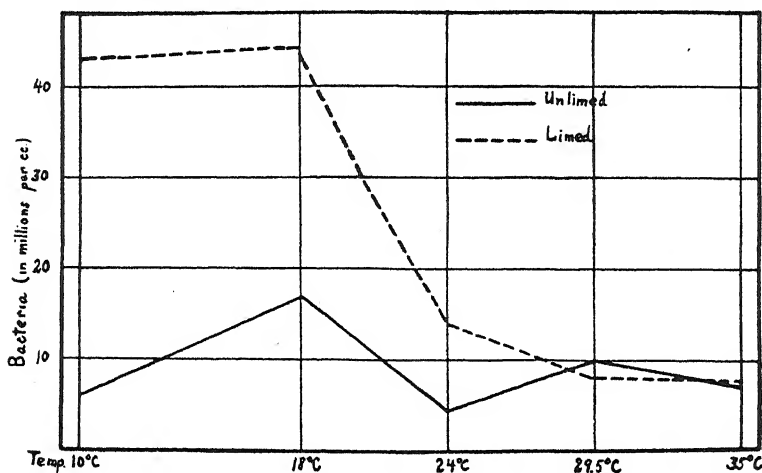
FIG. 1. AVERAGE TOTAL COUNTS OF BACTERIA IN LIMED AND UNLIMED FRESH SOLIDS DIGESTING AT DIFFERENT TEMPERATURES FOR 108 DAYS

albumen digesters, hydrogen sulfide producers, nitrate reducers and lactose fermenters. The composition of the media and the procedure involved are described elsewhere in detail.[4] The analyses were made, at first, twice a week, and, after two weeks, only once a week, for a period of ten weeks.

TOTAL PLATE COUNTS

The trend of the bacterial numbers in the unlimed materials incubated at different temperatures followed the same course

[4] Hotchkiss, Margaret. Bacteriological studies on the Imhoff tanks and sprinkling filter bed. Bul. 390, N. J. Agr. Exp. Sta. p. 49–68, 1923.

observed before in the digestion of solids, namely, a very sudden drop to a more or less uniform level which gradually rises again as digestion becomes complete. The average numbers are represented in figure 1. It can be seen that both the limed and unlimed materials at 24°, 29.5° and 35°C. had lower average numbers than at the two lower temperatures. The same kind
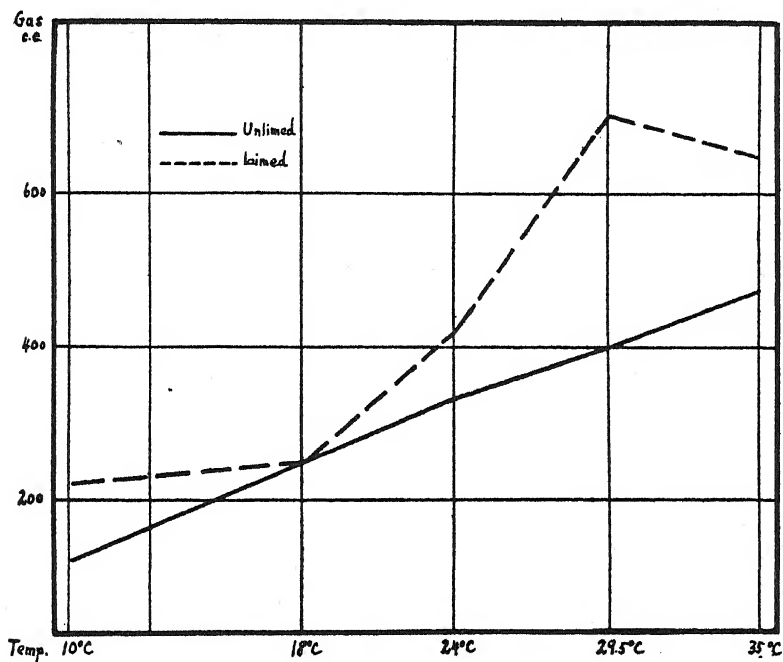
Fig. 2. Total Gas Production from Fresh Solids Digesting at Different Temperatures (108 Days)

of relationship holds when the numbers are computed on the basis of organic matter present in the sludge. When lime was added, the initial reduction in the numbers of organisms did not take place at 10° and 18°C. Lime, thus, retarded the initial reduction of the organisms which ordinarily find the medium at this stage unfavorable. Lime at higher temperatures however, did not prevent the reduction of the organisms.

The initial reduction of the numbers of bacteria might be due to the effect of high acidity produced in the first few days of digestion causing the pH value to drop from 6.4 to 5.2. The intestinal organisms in the fresh solids finding the medium unfavorable for their growth, are under these conditions greatly reduced in numbers. Low temperatures tend to prolong the life of the organisms and retard the rate of activities. The small amounts of organic acids produced at low temperatures when neutralized with lime tend to prolong the life of the organisms to a greater extent than would be the case without neutralization.
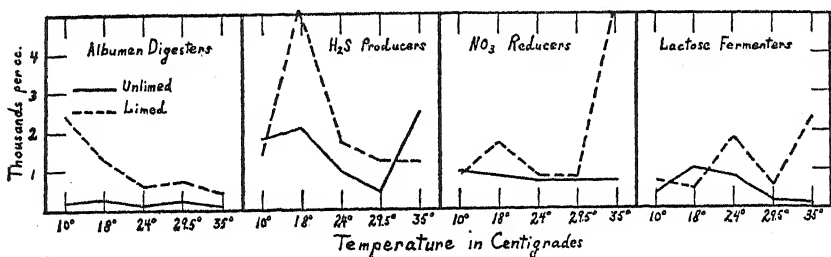
FIG. 3. AVERAGE NUMBERS OF ALBUMEN DIGESTERS, H2S PRODUCERS, NO3 REDUCERS AND LACTOSE FERMENTERS IN LIMED AND UNLIMES FRESH SOLIDS DIGESTING AT DIFFERENT TEMPERATURES FOR 108 DAYS

When the test was completed, after 108 days, the maximum gas production was obtained from material incubated at higher temperatures (fig. 2). Thus, high numbers of total organisms does not necessarily mean better digestion or greater rate of activity. Certain active groups of organisms, the numbers of which are over-shadowed by the presence of forms less active, must be responsible for the greater rate of digestion at high temperatures and for the greater volume of gas production. It is evident that the total plate counts do not serve as an index of digestion under all conditions.

PHYSIOLOGICAL GROUPS

The materials incubated at different temperatures showed large and erratic fluctuations in the numbers of the different physiological groups, which could not be correlated with the

actual processes of digestion. Therefore the numbers of these organisms were averaged and are represented in figure 3.

It will be observed that the average numbers of the different groups of organisms was not materially affected by the different temperatures of incubation with the exception of the numbers of hydrogen sulfide producers which were depressed at temperatures of 24° and 29.5°C. The numbers of the different groups of organisms, then, cannot be taken as an index of digestion, since the materials at the higher temperatures, digested much more rapidly than those at the lower temperatures. Lime on the other hand has a decided influence on the average numbers of these organisms. With only a few exceptions the average numbers of the different groups of organisms were higher for the limed material than for the corresponding unlimed material. However, even with the limed material the numbers of the different groups cannot be used as an index of digestion. In the case of albumen digesters and hydrogen sulfide producers the numbers in the material incubated at lower temperatures were higher than in those digesting at higher temperatures. In other words, the numbers of these different groups do not give a true picture of either the type or the rate of digestion. It may be further noted that whereas odors with limed materials were less than with unlimed, the H_2S producers were higher in all the limed materials. It would seem then that the organisms classed as H_2S producers by this physiological test are not responsible for the production of H_2S in the course of digestion.

The ability to digest albumen, to produce hydrogen sulfide from peptone, to reduce nitrates and to ferment lactose is not specific, e.g., is not a function of one or more species. Many organisms of widely different groups are capable of bringing about the above named changes. Further, the fact that these changes are observed in the culture medium is not an indication that the same organisms are bringing about similar changes in the sewage, since environmental conditions as well as food supply—two fundamental conditions determining the activities of micro-organisms—are widely different in a culture medium and in sewage.

ORGANISMS FERMENTING LACTOSE

Since the grouping of organisms into rough physiological groups as indicated above did not give a true index of the course of digestion and the organisms responsible for it, it became necessary to study these groups in greater detail and try to split them up into smaller groupings. For a start the lactose fermenting organisms were selected. It was realized that organisms of different physiological and taxonomic groups were included in the test for lactose fermentation, *e.g.*, intestinal organisms of the *B. coli* group, and anaerobic spore forming types.

After the test for gas formation in the lactose tubes was completed the tubes were incubated for an additional week in order to induce the sporulation of the spore-forming organisms. The tubes were then heated to 80°C. for fifteen minutes and 1 cc. of the contents transferred into fresh lactose tubes. It was assumed that within nine days most of the spore-forming organisms originally present in the sample in an inactive state would sporulate. Since the main question was to determine the numbers of the spore-forming organisms in an active and not in the spore stage, it was essential to employ an indirect method to induce the organisms to sporulate. The mere presence of the spores of the organisms in the material would not attribute to them any essential rôle.

The results obtained by the above procedure for the latter part of the digestion period are represented in figure 4. The curves show that, at the lower temperatures, a wide divergence existed between the numbers of total lactose formers and of spore-forming lactose fermenters. As the temperature increased, this divergence became smaller, so that in the two or three higher temperatures employed in the experiment the numbers of spore-forming lactose fermenting organisms constituted the majority of the lactose fermenting organisms. It would seem thus that we have here a first indication of a correlation between a certain group of organisms and digestion. It is quite probable that these spore-forming organisms belong to the genus Clostridium, the members of which group are known to be active proteolytic and carbohy-
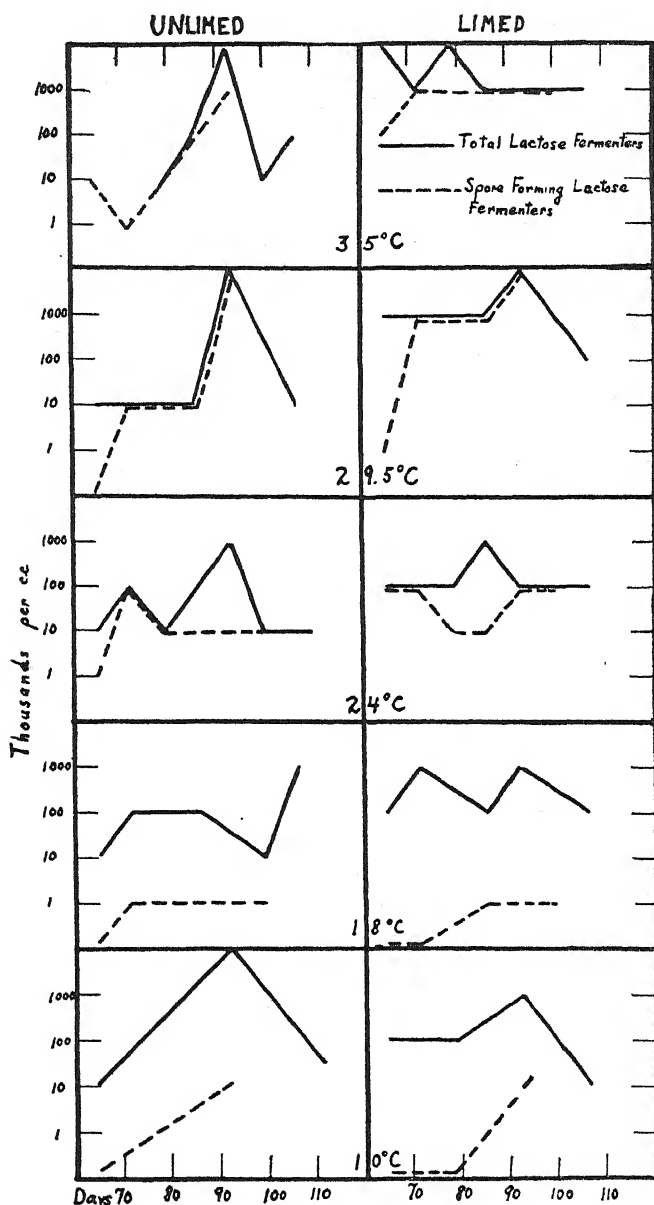
FIG. 4. COMPARISON OF TOTAL LACTOSE FERMENTING ORGANISMS AND SPORE
FORMING LACTOSE FERMENTING ORGANISMS IN LIMED AND UNLIMED FRESH
SOLIDS DIGESTING AT DIFFERENT TEMPERATURES FOR 108 DAYS

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drate fermenting organisms.   No claim is made that these spore-forming organisms are the sole cause of gassification or the only groups active during the last stage of digestion when gas production is greatest.

SUMMARY

Sewage solids subjected to different temperatures were incubated with and without additions of lime.   Total bacterial counts were made and the approximate numbers of different physiological groups determined.   The average number of the different groups of organisms was not affected by different temperatures, except in the case of H_2S producers, but the addition of lime influenced the average numbers.   A wide divergence was found between total numbers of lactose formers and spore-forming lactose fermenters.   With increase in temperature, the divergence became smaller so that, at the higher temperatures employed, the spore-forming lactose fermenting organisms constituted the majority of the lactose fermenting organisms, while the total numbers of organisms (plate counts) were greatly reduced.

A REVIEW OF THE DEVELOPMENT AND APPLICATION OF PHYSICAL AND CHEMICAL PRINCIPLES IN THE CULTIVATION OF OBLIGATELY ANAEROBIC BACTERIA

IVAN C. HALL

Department of Bacteriology and Public Health, University of Colorado School of Medicine, Denver, Colorado

The older methods of cultivating obligate anaerobes have been reviewed by Roux (1887), Novy (1893), Hunziker (1902), Matzuschita (1902) and Von Oettingen (1903). The work of Hunziker was especially valuable in its time but since then many important improvements in technic have been made.

The numerous methods that have been suggested for cultivating the obligately anaerobic bacteria involve, as a rule, a combination of one or more of the principles listed below under (1) reduction of oxygen tension, with one or more of those listed under (2) maintenance of reduced oxygen tension.

1. Reduction of oxygen tension
   A. Biological reduction
      Aerobe-anaerobic symbiosis
         Symbiont in medium
         Symbiont in air chamber
         Use of animal and plant tissues
   B. Physical reduction
      Boiling
      Evacuation
      Use of inert gases
   C. Chemical reduction
      Agent in air chamber
         Catalytic ignition of hydrogen
            and residual oxygen
         Reduction by phosphorus
         Reduction by iron compounds
         Reduction by alkaline-pyro-gallol
      Agent in medium

2. Maintenance of reduced oxygen tension
   A. Portion of medium sealed off
      Deep medium seals
      Insoluble liquid seals
      Mechanical seals
   B. Air chamber sealed
      Fusion of glass outlet
      Mechanical seals

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Since I have already presented the subject of biological reduction of oxygen tension in the cultivation of obligately anaerobic bacteria, with the various interesting practical applications and theoretical interpretations based thereon in "The Newer Knowledge of Bacteriology and Immunology,"[1] I shall restrict myself here to a discussion of the physical and chemical principles involved in the production and maintenance of anaerobiosis.

PHYSICAL REDUCTION OF OXYGEN TENSION—BOILING

Boiling is the simplest means of driving absorbed oxygen out of a culture medium. It was used by Pasteur (1861) and has been used by many investigators since. All that is necessary is to place the tube or flask containing the medium in a boiling water bath for a few minutes; the reduced solubility of gases at the temperature of boiling water and the fact that each escaping bubble of water vapor behaves as a vacuum toward any dissolved gas, explain the removal of oxygen by this means. Boiling may be used to drive oxygen out of semisolid, liquefiable solid, and liquid media in tubes or flasks and may be used in combination with other methods of reduction and various methods of sealing. Generally speaking, the medium should be cooled to about 45°C. before inoculating, though this is unnecessary for the sporulating anaerobes. Boiling is unsuitable as a means of oxygen tension reduction for surface methods of cultivation.

DEEP MEDIUM SEALS

For deep, semisolid or liquefiable solid media, boiling is all that is necessary to insure anaerobiosis, since reabsorption of oxygen generally fails to inhibit growth below a depth of 1 to 2 cm. This is the case with the deep agar and gelatin of Hesse (1885), Liborius (1886), Veillon and Zuber (1898), the deep brain medium of Von Hibler (1899), the cooked meat medium of Holman (1919), and the semisolid agar of Lignierès (1919) and others.

Deep glucose agar has been extensively used as a means of

[1] Edited by Dr. E. O. Jordan and Dr. I. S. Falk and published by the University of Chicago Press, 1928.

isolation of pure cultures.   To get at the colonies in the depths
it has been the practice to secure them by one of the following
methods: (a) aspiration into a capillary pipette, (b) cracking
the tube and its agar plug to expose the selected colony, (c) using
cylinders plugged at the lower end with a rubber stopper and
pushing the agar plug out into a sterile dish for inspection, as
advised by Burri (1902), or (d) expelling from the cotton plugged
end of an ordinary tube by heating the base so that expanding
steam forces the plug out as advised by Konrich (1914).   All are
practical means of isolation, in the case of well separated colo-
nies, except the last, providing aerobic organisms have been
eliminated first.

One of the obstacles in the use of deep glucose agar for isolation
is fragmentation by gas.   Veillon and Mazé (1910) claimed this
to be due mostly to hydrogen in view of the rapid diffusion of
carbon dioxide and proposed the addition of 0.1 per cent KNO_3
for the fixation of the hydrogen.   The equation supposed to
represent the reaction fails to balance as given and my own effort
to confirm the value of KNO_3 as a means of preventing cleavage
of glucose agar failed for pure cultures of *B. Welchii*, *B. botulinus*,
and *B. sporogenes*, though a satisfactory result was obtained with
Bacillus septicus.   But a better method of avoiding excessive
gas formation is to reduce the fermentable carbohydrate content
of the medium to a minimum, say 0.1 per cent, by using meat
infusion 1 per cent agar without added sugar.

It is well to melt the agar surface, above the colony to be
picked, with a hot spatula to avoid organisms not belonging to
that colony.   Theoretically the temperature attained upon
the melted surface cannot exceed the boiling point, which is
known to be insufficient to kill many spores, but practically
there is rarely any contamination of cultures with this technic.
The selected colony can best be removed by means of a heavy
platinum wire, flattened at the end and bent into a tiny hook.
This is preferable to aspiration by means of a capillary tube,
as sometimes advocated.

Esmarch (1886) adapted his roll cultures for isolation of aerobes
to the isolation of anaerobes by filling the central space with a

core of sterile agar, and Marpmann (1898) suggested forcing the inoculated agar into a thin sheet by means of a small sterile test tube inside a larger one containing the medium. The paraffin seal used by the latter is quite unnecessary. The same may be said of Friel's (1917) use of a Bunsen valve to exclude air after boiling liquid media. How his complicated apparatus can be adapted to the isolation of pure colonies from roll or slope agar cultures is hard to understand.

The use of slender tubes by Vignal (1887), Van Senus (1892), and Streng (1903) to facilitate the examination and removal of isolated colonies in the depths, relies upon the same principle of exclusion by a deep medium; the incidental reinforcement of the seal by fusion of the glass in the case of the first two is only a matter of convenience and not of necessity.

Sufficiently deep layers of liquid media also frequently yield satisfactory growth, which is largely the explanation of the successful use of the Smith fermentation tube (1893) (1899) (1905) for anaerobic cultures, except, of course, where sterile tissues are added to the media.

Rosenthal (1906) showed a direct relation between the diameter of the tube and the required depth of liquid medium necessary to insure anaerobic growth, dependent probably upon the factor of more active convection in wide tubes leading to greater rapidity of oxygen absorption.

There is an element of uncertainty in relying merely upon the depth of *liquid* media to exclude oxygen, however, except where the depth is considerable and the inoculum large, as in the production of toxin in large flasks according to Anderson and Leake (1915); ordinarily, some other seal is desirable. Kovac's (1924) claim of aerobic growth of obligately anaerobic bacteria in 10 per cent peptone broth seems to me to depend upon nothing but the increased viscosity of the broth due to the high peptone content.

INSOLUBLE LIQUID SEALS

Insoluble liquid seals have been used in the form of oil by Pasteur (1863), lanolin by Rosenthal (1902) (1903), vaseline by

Nicolle (1902) and Beattie (1906), and paraffin by Kasparec (1896) and Park (1901).

The idea of using mercury for sealing the bend in a fermentation tube for anaerobic cultures occurred to Smith (1890), but, according to a recent personal communication, was never tried by him.   In our hands, controlled tests of a variety of obligate anaerobes in glucose broth so sealed in fermentation tubes uniformly failed to permit growth, owing, no doubt, to the formation of germicidal salts of mercury in the medium.

In the use of oils, greases, and waxes, the seal is sometimes placed in position before sterilization, at other times after separate sterilization and inoculation; there is really no objection to the first method, but in the case of solid or semisolid seals, the organisms must be placed in the medium previous to the setting of the seal.   Valid objections to all oil seals are their messiness, the difficulty of cleaning glassware, their interference with good staining, and, above all, a serious question as to the efficiency of liquid oils in excluding oxygen.

Vernon (1907) pointed out that olive oil and codliver oil dissolve about 4.5 times as much oxygen at 37°C. as does water, and Wolf, McGill and Harris (1917) suggested that the only real value of oil in anaerobic cultures is a possible reduction of convection currents.

Mineral oils have been preferred to saponifiable oils, as being relatively indifferent chemically.   With a mineral oil, the writer has shown, in comparison with the constricted tube and marble seal (Hall, 1915), that growth is delayed unless the inoculum be large.   In these experiments the combination of oil in the constricted tube with a marble seal showed that such delay was not due to antiseptic properties of the oil, and I was forced to conclude that oil was inadequate as a seal.   With methylene blue as a criterion of anaerobiosis, the color returns to a decolorized solution covered with several centimeters of oil almost as quickly as without oil (Hall, 1920).   This was true whether the oil had a boiling point of 300°C. as in the case of white mineral oil, or of 100°C. in the case of heptane.   Fermi and Bassu (1904) were also unable, by prolonged boiling, to prevent the subsequent

darkening of alkaline-pyrogallol in media under liquid paraffin. It seems that a possible greater solubility of air in oil is partly responsible for these results but in contrast with Wolf, McGill and Harris (1917) I am inclined to attribute the return of color in reduced methylene blue solution under oil to diffusion currents in the liquid oil.

The inefficacy of the usual white mineral oil, both as regards the growth of obligate anaerobes and the protection of decolorized methylene blue, was first thought to be related to its relatively high boiling point (295° to 310°C.), but comparative tests with *B. tetani* in glucose broth layered to a depth of 2 cm. with four hydrocarbons of different boiling points, namely heptane (95° to 100°C.), xylol 137° to 140°C.), white mineral oil (295° to 310°C.), and paraffin (300° to 315°C.), showed the fallacy of this idea, for no growth was secured under heptane or xylol, while mineral oil was second only to the solid paraffin.

It was easily shown by means of heptane and xylol, layered over similar media in constricted tubes with marble seals, that there was no evidence of antiseptic action for the relatively large inocula used. The methylene blue tests definitely showed the advantage of paraffin over the liquid hydrocarbons, even in one-tenth the thickness of the latter, and both these, and the cultural tests with *B. tetani* and other anaerobes, showed that 0.25 cm. of paraffin is a sufficient seal if it remains intact.

What then is the explanation of the widespread and more or less successful use of oil as a seal for anaerobic cultures? The factor of increased depth of liquid may be partly responsible, coupled with the practice of boiling out of media, use of reducing and porous substances, and large inoculations. Larson, Cantwell, and Hartzell (1919) suggested that reduction of surface tension by the layer of oil may be an important factor, but their viewpoint awaits confirmation.

In case of the solid or semisolid greases or waxes, diffusion currents are impossible in them when they are cold, and decolorized methylene blue fails to regain its color in media so protected. Beattie (1916) pointed out the value of vaseline as a rough means of gas measurement for anaerobes. Vaseline has an advantage

in that it does not separate from the container, as does paraffin, which latter fault Kasparec (1896) very ingeniously sought to remedy by tapering the neck of his flask; the accessory bulb to hold the liquefied paraffin in his method, is however impracticable and unnecessary.

Dick (1918) used paraffin as a seal in the isolation of pure cultures of anaerobes. The material under examination was streaked out upon the surface of hardened agar in a Petri dish, covered with sterile agar, and, when this was hard, the sterile melted paraffin was poured on. The method gives only submerged colonies, of course. Northrup (1919) suggested coloring the paraffin with water-insoluble wax-soluble dyes such as Sudan III or Scharlach Roth to facilitate picking out the colonies. Fine charcoal was also found useful.

I have found a mixture of equal parts of vaseline and paraffin preferable to either alone, and we have used this compound under the coined name "vaspar" for liquid media such as broth, milk and gelatin, particularly when long time observations were required, as of *B. putrificus*. Such a seal effectively prevents evaporation, as well as the re-absorption of oxygen.

MECHANICAL SEALS

Some of the most efficacious devices for the exclusion of air from boiled media are to be found in this group.

Nencki (1879) used a constricted tube in which a glass rod was pushed through a rubber stopper to seal off the base after inoculation. This device might have been successful had he not made it too complicated by an attempt to combine the use of evacuation and chemical reduction with alkaline-pyrogallol.

A mechanical type of seal, in addition to the depth of the medium, was invented by Wright (1900) in the form of a glass and rubber tubing device placed inside the culture tube. The apparatus is positive in action and successful even if cumbersome, as shown by Tuck (1903). I have used it successfully.

Another type of mechanical seal is Durham's (1898) (1900) fermentation tube and the similar, though vastly more complicated arrangement, of Epstein (1898) for the measurement of gas

production. Neither of these lends itself to easy and certain inoculation of the anaerobic chamber, though motile bacteria are likely to find their way past such seals more readily than air, due to the active chemiotactic responses of the bacteria, which drive them away from oxygen, so that successful growth may occur even when the inoculation is made at the surface.

One of the simplest, and at the same time, most satisfactory devices using a mechanical seal, is the constricted tube described by the writer in 1915, in which a marble, porcelain biconvex disc, or round cover slip, rests upon a constriction in a tube to seal the liquid below from that above. Kendall, Cook and Ryan adapted the idea to flasks in order to study the metabolism of anaerobes (1921) and Holman (1922) used a small test tube in place of the marble seal, but the test tube seal is quite unsatisfactory as it prevents access to the anaerobic culture. I also devised a special constricted tube with marble seal for anaerobic fermentation tests (1921). It is surprising how effectively the marble seal prevents re-absorption of oxygen into a liquid culture medium from which the air has been driven by boiling. Methylene blue remains decolorized for days below the seal although under suitable conditions of alkalinity and organic content in the solution, the color returns above the seal within a few minutes. Obligately anaerobic bacteria produce growth in suitable media under the marble seal even when incredibly small inocula are introduced. The constricted tube was designed primarily for making combined aerobic (above the seal) and anaerobic (below the seal) tests of biologic products but its greatest usefulness has proven to lie in providing suitable conditions for primary cultures of pathologic materials suspected of containing anaerobes, in the preparation of small quantities of anaerobic fluid cultures for inoculation of animals, and for serologic tests, and in the differential diagnosis and identification of anaerobes by cultural tests in meat, milk, gelatin, and fermentation media. The use of a marble seal has also been adapted to the test for relative stability in sewage by Pomeroy and Stone (1928).

Another simple method involves the use of sand as a seal in the bend of the Smith fermentation tube, or even in the base of

an ordinary tube filled with broth or other liquid medium. Using white sea sand, a black pigment-like substance is deposited by some cultures, which may prove to be of differential value. It is well, if the media be not freshly sterilized, to heat for ten to fifteen minutes in the steaming Arnold sterilizer and cool immediately prior to inoculation, though satisfactory growth may often be obtained without this precaution.

It is to be assumed that sand used as a seal in fermentation tubes acts purely as a mechanical deterrent to re-absorption of oxygen; it is probable, also, that this mechanical factor is partly responsible for the results obtained when the bend is filled with tissues as in Smith's original recommendation (1890).

It is difficult to discuss the problems of cultivation of anaerobic microörganisms in the depths of media without becoming involved in the theoretical aspects of the subject. Many anaerobes, in their growth, produce gases, such as hydrogen, carbon-dioxide, and methane, and these, in their escape, remove any traces of oxygen left in the medium exactly as bubbles of water vapor remove gases in the initial boiling. For that reason the inhibitive action of oxygen is most evident in young cultures, as Burri and Kürsteiner (1908) have mentioned. For the growth of anaerobic gas-formers in deep media, therefore, it is only necessary to provide for the initiation of growth in a secluded portion of the medium; the escape of gas soon deoxidizes the whole, so that growth occurs throughout, in the case of liquid media. These facts have an interesting bearing on the so-called aerobic growth of obligate anaerobes through the addition of various porous substances; Douglas, Fleming and Colebrook (1917) have shown the value of these substances to lie principally in the provision of anaerobic interstices for the initiation of development. The successful use of spongy platinum in liquid media for the growth of *B. botulinus*, *B. oedematis*, *B. Chauvoei*, and *B. putrificus* by Pfuhl (1907) is better explained thus than upon the idea of its acting as an oxygen absorbant. All such porous substances actually provide a mechanical seal in the liquid against rapid re-absorption of oxygen. An undeniable advantage of deep media containing mechanical seals for initial cultures is that a wide range

of oxygen tensions is provided where various and unknown types of organisms may be present.

While mechanical seals, other than those furnished by semi-solid or porous constituents of media, have been relatively little used for liquid media, they have found considerable utility in the case of solid media, especially agar, for the study and, in some cases, isolation, of single colonies in the depths. These methods are eminently suitable for the first purpose. Thus Koch used thin plates of isinglass and Sanfelice (1893), Streng (1903), and Liefmann (1908) thin pieces of glass, on the surface of plate cultures for the cultivation of anaerobes. Braatz (1895) also showed the inhibitory effect of anaerobiosis so obtained upon certain obligate aerobes. The use of agar in the special plate of Streng (1903) or between the inverted halves of a Petri dish as prescribed by Fehrs and Sachs-Müke (1908) and by Krumwiede and Pratt (1913) is merely an extension of this principle, combining, in the last case, the additional use of paraffin.

Many have used containers with outlets which might be sealed. Thus Pasteur (1861) dipped the end of such a tube under mercury, or sealed a constricted portion by fusion after inoculation. Biffi (1907) and McLeod and Soga (1914) fused the outlet tubes extending through rubber stoppers used to close their containers; though the inverted vial with the liquid medium of the former and the complete filling of the container of the latter with semi-solid medium, together with the use of sterile tissues in the depths, might be considered to provide independently adequate means of anaerobiosis. Reuschel (1906) used a pair of hemostatic forceps, and Moragas and Roig (1915) a pinch cock, for compressing a rubber tube outlet attached to a partially evacuated container. The use of the latter for anaerobic blood cultures utilizes the partial vacuum not only as a means of anaerobiosis but also for aspiration from the vein, a principle already developed in the United States by Keidel (1912).

EVACUATION

Evacuation is also one of the earliest methods, and one that may conveniently be combined with boiling to eliminate oxygen

from semisolid or liquid media *after* inoculation, without destroying the organisms, since the boiling point is lowered by the reduced air pressure. The first use of evacuation is ascribed to Pasteur (Hunziker, 1902), and it was also utilized by Nencki (1879).

Various forms of vacuum pump have been used; our knowledge of those utilized by the earlier investigators is somewhat obscure, but Roux (1887) and Schottelius (1887) used mercury aspirators, Roux (1887), Durham (1896), Klein (1898), Süpfle (1909), Penfold (1910), and Holker (1918), water aspirators, and Meyer (1905) and Dack, Starin, and Werner (1927), mechanical air pumps. It is customary to rely upon some type of manometer, either the mercury type, which is preferable, or a mechanical vacuum gauge, for measurement of the pressure. Zupinski (1898) suggested a form of container directly attachable to a mercury manometer, in which the fall of the mercury itself produced a vacuum.

The vacuum method has been a favorite one for the study of the limits of oxygen tension in which bacteria grow, as for example, by Süpfle (1909) with cholera; Rosenthal (1906) has apparently shown the possibility of changing these limits for certain anaerobes. Yet we have as yet no adequate information on the oxygen tolerance of the obligate anaerobes generally, although Dack, Starin, and Werner (1927) have shown that while oxygen pressures equivalent to 5 cm. of mercury or more inhibit the growth of *B. botulinus* and *B. sporogenes*, growth can be secured regularly at pressures of 4 cm. or less. In computing the oxygen tensions it is, of course, necessary as Penfold (1910) pointed out in his anaerobic cultivation of intestinal aerobes, to allow for the vapor tension of water.

Methods of maintenance of vacuum appear to have been restricted mainly to the sealing of the air chamber, though there is no reason to think the sealing off of a portion of the medium would not be equally efficacious, as in boiling, where surface cultures are not contemplated.

Containers for anaerobic cultures in vacuo have been of two general types, first, those in which the vessel containing the medium is itself sealed by fusion of a connecting tube as by Pas-

teur and Roux (1887) and as according to Gruber (1887), who prepared Esmarch roll cultures in this manner, or by means of a stop or pinch cock; and second, those in which a vessel of moderate size is made to contain one or more smaller tubes or plates holding the medium. When these involve tapered ground glass stoppers there is great danger of jamming and subsequent breakage. Gage and Spurr (1913) have undertaken to obviate this difficulty by replacing the ordinary glass covers of museum jars with brass covers provided with two small metal pinch-cocks. But it is not an easy matter to construct an air tight chamber, impervious to leakage, so that the vacuum method is rarely used alone. For this reason the historical development of such containers is best reserved mainly for discussion under the next heading.

USE OF INERT GASES

Exhaustion of oxygen by the use of inert gases is also dependent upon principles similar to those involved in boiling and evacuation, in that each bubble of introduced gas carries out its quota of oxygen. The gas may be passed through the media or it may be passed over the surface. The former is more efficacious in the removal of oxygen, though obviously not applicable to the cultivation of surface colonies, which requires the latter method. But in thin layers of media, such as are generally used for this purpose, the diffusion of absorbed gases due to variations in partial pressures at the surface probably occurs quite rapidly.

Evacuation and the use of inert gases are most successful when combined, since both are applicable to media containing heat-coagulable proteins and the use of a gas obviates the difficulty of attempting to maintain a high vacuum. Moreover, the alternate application of vacuum and gas facilitates a very complete reduction of oxygen tension. Again, Pasteur was the pioneer in the use of carbon dioxide. Carbon dioxide is now known to be somewhat deleterious through the action of the weak acid formed with water, which inhibits the growth of some organisms, e.g., blackleg bacillus, according to Kitasato (1889). It is an enticing conjecture that Pasteur was led to the use of carbon

dioxide not alone by the ease with which it may be prepared, but also because in fermenting liquors, carbon dioxide is one of the products of yeast fermentation, whose constant escape tends to remove free oxygen, thus producing a condition of true anaerobiosis.   Dack, Starin, and Werner (1927) found that *B. botulinus* and *B. sporogenes* grew, and the former produced strong toxin, in carbon dioxide atmospheres equivalent to 50 cm. mercury.

But, in the early development of technic, other gases ultimately supplanted carbon dioxide, notably hydrogen, whose first use was also ascribed to Pasteur in 1861, by Roux (1887) and by Hunziker (1902), although I have been unable to confirm this. Certainly Hauser in 1885 was not the first, as mentioned both by Heim (1891) and Hiss and Zinsser, nor Lackowicz and Nencki (1884) as mentioned by Jungano and Distaso (1910), for Gunning (1877) (1878) (1879) referred to Gratama, one of his students in the University of Amsterdam, as having cultivated putrefactive anaerobes in oxygen free atmospheres of nitrogen and hydrogen. Nitrogen has never been much used as a manufactured gas for inducing anaerobiosis, though anaerobes grow well in it; hydrogen, on the other hand, has been greatly exploited.   Lackowicz and Nencki (1884) generated hydrogen from sulfuric acid and iron, but it is generally produced now through the action of sulfuric acid on granulated zinc in the presence of a catalyser, such as platinum chloride, in a Kipp or other generator.   On account of probable impurities in the commercial materials, the gas should be passed through a series of washing bottles, containing respectively 10 per cent lead acetate to remove sulfuretted hydrogen, 10 per cent silver nitrate to remove arseniuretted hydrogen, and a mixture of 10 per cent pyrogallic acid and 10 per cent lye to remove traces of oxygen.   Even so, it is difficult to obtain a thoroughly satisfactory product.

A more modern method has been suggested in the use of steel cylinders of compressed hydrogen, produced by electrolysis of water; this would seem to be ideal, but I have given it a thorough trial in an effort to secure surface colonies of various obligate anaerobes with indifferent success.   The reason for this failure is unknown; it may be due to impurities.

Durham (1896) ingeniously combined the generation of hydrogen with the absorption of oxygen by sodium hydroxide and pyrogallic acid by allowing water to act upon an amalgam of sodium and mercury. He showed that the naphtha, which clings to the metallic sodium when taken out of it, and vaporized during the process, has no deleterious action on the tetanus bacillus.

The recent use of spongy platinum and palladium as catalysers to facilitate the hydrogenation of the last traces of oxygen may be mentioned here, but their discussion properly belongs under the heading of chemical reduction.

Illuminating gas was suggested as a convenient substitute for hydrogen by Würtz and Foureur (1889). Bullock (1900), Wilson (1917) and Holker (1920) have invented special chambers for Petri dish cultures, in which they claim to have obtained surface colonies of obligate anaerobes in hydrogen and coal gas. The experiments of Ferran (1898) in acclimating the tetanus bacillus to an increased oxygen pressure by successive cultivation in atmospheres of decreasing proportions of acetylene and air are interesting in this connection, as well as in relation to his claims of aerobic growth of this obligate anaerobe; but they are of doubtful value. However, Kladakis (1890) and more recently Ludwig (1918) found illuminating gas to have a disinfectant action on bacteria. No doubt much depends upon the particular kind of gas used. The convenience which the laboratory jets offer warrants a full trial in each locality. In Berkeley, California, where gas produced from crude oil is supplied, some promising results were obtained with surface cultures in plates manipulated according to Jones' (1916) method; incomplete success at first was attributed to a slight acidity and the removal of this by passing the gas through a lye solution gave very satisfactory results.

The remarks relating to seals for the vacuum method, which is so often combined with the use of an inert gas, apply with equal force in each case, only that where no attempt is made to produce a vacuum there is less danger of ground glass stoppers jamming.

Various types of containers have been used, including tubes, flasks, special plates, and containers for ordinary Petri dishes.

FLASKS AND TUBES AS CONTAINERS

Pasteur (1861) passed carbon dioxide into liquid media in flasks, the excess gas escaping through a mercury seal. Lackowicz and Nencki (1884) did the same thing with hydrogen. Hesse (1892) passed a stream of hydrogen through mercury into an inverted tube of solid slanted medium, while Hewlett (1894) used a yeast flask provided with two tubes, one as a hydrogen inlet and the other as an outlet dipping into a dish of mercury.

Liborius (1886) used a constricted tube, into the liquid or slanted solid medium of which, hydrogen was passed by means of a small tube sealed into the side, this and the neck of the tube being finally sealed off by fusion, inoculation having been already accomplished by means of a capillary tube passing through the cotton stopper above the neck.

Fränkel (1888), eliminating the neck and side tube, provided a rubber stoppered tube of deep medium with tubes arranged like those of a Wolff bottle, which might be fused when saturation had occurred. Petri and Maaszen (1893) also used the same idea in tubes and flasks with tubulature sealed in.

Saturation of media in tubes or flasks by means of hydrogen or carbon dioxide passed through a capillary tube in the cotton stopper and sealing by fusion of a constricted portion of the container became the subject of an amusing polemic between Heim (1891) (1892) and Ogata (1892), in which Van Senus (1892) finally claimed priority for himself as the inventor in 1890.

These methods appear to have been applied only to deep cultures until Ewell (1897), in a procedure similar to Fränkel's, combining the use of a vacuum and relying upon a warm water bath to maintain fluidity of the agar till after fusing the outlet and inlet, prepared roll cultures after the manner of Esmarch for isolated surface colonies. However, Fuchs (1890) had previously cultivated an obligate anaerobe on the surface of slanted coagulated ox serum in an inverted tube filled with hydrogen and sealed immediately by means of a paraffined rubber stopper, and we have already referred to the work of Hesse (1892).

We might mention here the use of capillary tubes of hydrogen

under slight negative pressure by Pende and Viviani (1907) as a method of somewhat doubtful value in cultivating anaerobes in this gas for any purpose whatsoever.

Perhaps the most complicated development of methods for the culture of anaerobic microörganisms in tubes or flasks with hydrogen has been reached in those procedures of Noguchi (1911) for the growth of various spirochaetes, in which the use of an inert gas is combined with evacuation, a reducing agent (alkaline pyrogallol) in the chamber and a reducing agent (sterile tissue) in the medium as a means of oxygen reduction and the use of a sealed chamber, deep medium and oil as a means of maintenance. Having once shown the possibility of cultivating the *Treponema pallidum*, it was only natural that the complex procedure should be simplified, as, for example, by Baeslack (1913), who eliminated the oil, and Bronfenbrenner (1914), who eliminated the hydrogen and the closed chamber seal, but retained the oil.

SELF CONTAINED PLATING DEVICES

Most of the containers so far mentioned failed to provide for surface cultivation; it is the special aim of plating devices to contribute to this end, with a view to the isolation of well-separated colonies and pure cultures. As a form of container intermediate between flasks and plates may be mentioned the flattened flasks of Kitasato (1889), and Roth (1893), with inlet tube for hydrogen and cotton plug sealed with paraffin at the conclusion of the operation. Roth, later, eliminated the tube as a part of the flask, finding it an unnecessary complication, for which a tube passing through the cotton stopper might be substituted just as well. Berner (1904) also invented a flattened flask with two gas tubes, one provided with a ground glass stopper, the other with a ground glass cock, a combination almost impossible to sterilize without breakage.

Special types of plates with matched perforations for the passage of gas were invented independently by Gabritschewsky (1891) and Kamen (1892); sealing was accomplished by twisting the holes out of alignment, the former providing also for the use of a chemical reducing agent, alkaline-pyrogallol.

Migula (1895) used two glass dishes, one being provided with a tube for the passage of hydrogen which was allowed to escape through the hydrocarbon oil used in the larger one for a seal. Various other forms of double plates for paraffin sealing and tubulature for gas were invented about this time by Beck (1897), Epstein (1900), and Bombici (1902); all are open to the objections of their high initial cost as unusual apparatus and their great fragility. A more promising device of this general class was that of Jones (1916), consisting of a turned iron base with inlet and outlet holes for gas and a circular trough on top, into which a streaked Petri dish might be luted by means of paraffin previous to sweeping out the oxygen by means of hydrogen, the gas tubes being sealed by fusion thereafter. The writer had indifferent success with the method of Jones in the use of hydrogen gas from a Kipp apparatus but this was considerably improved in the use of gas supplied from the laboratory taps, as already described.

The principal objection to all such devices is the difficulty of obtaining them, which rests in the final analysis upon the fact that none has proven of such outstanding superiority as to make commercial production feasible.

JAR-LIKE CONTAINERS

Coincident with the development of these special plating devices for single cultures, there have been invented various methods whereby several plate cultures might be placed in a single container, usually a bell or jar.

Liborius (1886) combined the use of evacuation and hydrogen in a bell jar, provided with a rubber tube inlet and outlet and pinchcocks for sealing. This jar was held down on a rubber ring by means of clamps and provided for surface cultures on several agar plates at once. Variations, but not improvements, were introduced by Blücher (1890) and Botkin (1890). The former inverted a lead-weighted bell in a deep dish of glycerin, upon which the dish of medium was floated, hydrogen being introduced under pressure at the top of the bell through an inlet containing cotton, which was afterwards sealed by means of glycerin poured into the clamped end of a rubber tube; the latter used paraffin

as better than glycerin for sealing the base of the bell, introduced his gas through U tubes passing under the lips of the bell, and included a rack to hold the dishes. Botkin's bell was sterilized by means of corrosive sublimate solution and dried, first with alkali and then ether. The same general procedure was followed essentially by Hesse (1892) except for the use of a mercury seal instead of paraffin for the base of the bell.

The well known Novy jar was first described in 1893 and again in 1894. It provides a base with removable cap, containing a glass cock ground in, and carrying both inlet and outlet tubes. It has been used as a combination method for evacuation alternated with hydrogen or other gases. The stoppers of Novy jars in their present form almost invariably jam under vacuo. It is not surprising therefore that other forms of apparatus continued to be suggested, such as Hewlett's chamber for plate cultures (1894), with two tubes providing mercury valve seals, and Lubinski's jar with sealed in tubulature for oil valves (1894). Zettnow (1894) and Mereschowsky (1903) also contrived cheap metal containers to be used with hydrogen, sealed in by paraffin, in connection with alkaline-pyrogallol as a reducing agent, and Kedrowsky (1895) used a crystallizing dish on whose ground glass cover plate, at diametrically opposite points, perforations to permit the entrance and exit of gas could be turned out of alignment to perfect the seal, the ground glass surface having been smeared with vaseline. Such a plate could be made to contain the medium, as in the case of the methods of Gabritschewsky (1891) and Kamen (1892), or used with one or more enclosed Petri dishes. Durham (1896) devised a system of tubes and museum jars covered with an iron plate clamped down, and generated hydrogen by allowing a soda solution to act upon pyrogallic acid and sodium amalgam, an ingenious but clumsy method, of doubtful value. Petri (1900) advocated a plate and bell jar into which hydrogen could be passed through tubes in the rubber stopper in the top and in which alkaline-pyrogallol served both as a reducing agent and a chemical criterion of anaerobiosis. Bullock (1900) also used a bell jar with glass stopcocks sealed into the top, and Bordet (1904) adapted a desiccator to the same purposes, while

Gage and Spurr (1913) proposed an ordinary candy jar with a metal plate, perforated and provided with metal stopcocks, to avoid the breakage so often encountered with glass cocks, for passage of the gas. In 1917, Wilson devised a special base with inlet and outlet tubes to accommodate a bell jar and several Petri dishes.

Several modifications of the jar principle have been made in which there is no use of inert gases, reliance being placed on other means of oxygen tension reduction, such as the use of chemical reducing agents; these are discussed in their proper places.

The possible value of having a combination of methods, such as deep media with reducing agents, vacuum, inert gas, and chemical absorption, was exemplified in the work of Laing (1904) with several anaerobic bacteria, and more especially of Noguchi (1911), in cultivating the *Treponema pallidum*.

It seems to me that the use of inert gases is only justified for securing surface growth of the obligate anaerobes, whether for isolation or for observing the form of the colonies or their action upon certain media, since, as already pointed out, deep methods of cultivation or methods with mechanical or insoluble liquid (i.e. wax) seals are entirely adequate for purposes other than surface cultivation. The valid objections to surface cultures for isolation have been clearly stated in another place (Hall, 1920). When surface cultures are desired, it is simpler and preferable from a practical viewpoint to rely either wholly or in part upon chemical methods of reducing the oxygen tension.

CHEMICAL REDUCTION OF OXYGEN TENSION

Chemical reduction of oxygen tension provides some of the most useful means of cultivating obligate anaerobes. Chemical reducing agents may be used in two ways, either in a closed chamber but not in contact with the cultures, or in the media themselves.

CHEMICAL REDUCING AGENT IN CLOSED AIR CHAMBER

Use of a catalyser to ignite hydrogen and residual oxygen

During the war a method was developed by Laidlaw (1915) for cultivating obligate anaerobes upon the surface of solid media,

which combined the use of an inert gas and chemical reduction, depending upon the well known fact that spongy platinum absorbs an inflammable gas with sufficient generation of heat to ignite the gas in the presence of oxygen. The idea was first applied in tubes containing slanted media, or sometimes liquid media, provided with stoppers carrying a small mass of spongy platinum mounted upon a wire. The culture medium was inoculated in the usual manner. The cotton stopper was then removed and a small jet of hydrogen from a generator was led into the tube for a few minutes. The spongy platinum was then slightly warmed and quickly placed in the tube; if successful it would immediately glow until all of the remaining oxygen was consumed. Laidlaw also tried spongy palladium without advantage, though McIntosh and Fildes (1916) found palladium cheaper in cost, more efficacious in operation, and superior to all other methods for the surface isolation of pure cultures. The method was also used successfully by Stoddard (1918) but my own experiences with it at that time were not successful, owing to the invariable occurrence of accidents, either failure to ignite the hydrogen or violent explosions; no surface cultures could be obtained.

In 1917, Smillie devised tubes and jars into which hydrogen could be led and a platinized asbestos catalyser heated after closure. With this he claimed success, not only in cultivating the common anaerobic bacilli but also the "globoid bodies" of poliomyelitis. A similar device for use with palladinized asbestos was described in England by Fildes and McIntosh in 1921, while in America, improvements in the arrangement of the heating element were made by Brown (1921) to secure better contact of oxygen and hydrogen with the catalyser and by Brown (1922) and Richardson and Dozier (1922) to avoid explosions and to prevent cracking the glass covers by the heat. The most highly developed form of anaerobic jar is that of Wilson (1928) based upon the original ideas of Laidlaw and of Smillie, in the form of an all metal jar with provision for evacuation, inert gas, and catalytic reduction activated by electric current.

A slightly different trend manifested itself in the "central

station for catalysis" devised by Boez (1925) (1926) to which one or several containers of similar or different types might be attached for reduction of oxygen tension.

Use of phosphorus as a reducing agent

The earliest reference to the use of this vigorous reducing agent found by the writer is that of Gunning (1877) to the work of Gratama, already mentioned in connection with the use of hydrogen. Gratama cultivated bacteria in atmospheres considered to be oxygen-free because of the failure of phosphorus to ignite, thus using this element as a criterion of anaerobiosis rather than as a primary means of reducing oxygen pressure. Sellards (1904) appears to have been the first to utilize phosphorus for this purpose; he cultivated the tetanus bacillus in a hanging drop by this means and studied the effect of anaerobic growth upon the metabolic activities of certain aerobes, a number of which, for example, liquefy gelatin only aerobically. He found that certain precautions had to be followed in handling phosphorus, due to its poisonous and inflammable character and to a possible deleterious action of phosphoric acid upon the cultures, which could be prevented by the provision of an absorbing body of alkali. Surface cultivation of obligate anaerobes was not described by Sellards, but Bushnell (1922) succeeded with several species. There was some difficulty in the formation of deposits upon the surfaces of plates and cotton plugs and in the decolorization of red wax pencil labels, but these objections were overcome by covering the cultures with paper and using blue pencils. Bushnell found that it was unnecessary to use lye to absorb the fumes, water serving equally well. Bushnell's success was confirmed by Varney (1926) who, instead of fruit jars and fireless cookers, used glass museum jars with metal racks for the Petri dishes. Chapman (1928) found Varney's method useful in the selective isolation of *B. Welchii*.

Use of iron compounds as reducing agents

Drossback (1893) postulated the presence of anaerobic bacteria growing at 37°C. as the true criterion of water pollution and

proposed their detection by means of cultures in a desiccator sealed by a wax and oil mixture. From this desiccator it was proposed to absorb the oxygen by iron "oxydul" prepared by the action of strong lye on iron chloride. This work is unimportant, but the reducing action of iron and iron salts in culture media used for anaerobic organisms presents a promising field, in which important beginnings have already been made by Scott (1926).

Use of alkaline pyrogallol as a reducing agent

No chemical reducing agent has had a more extensive use in bacteriology or resulted in more kinds of fantastic apparatus for bacterial cultures than the mixture of strong alkali and pyrogallic acid. The vigorous avidity for oxygen possessed by such a mixture is well known, and, while the chemical textbooks are strangely silent on the question of the exact reactions involved, carbon monoxide, carbon dioxide, and acetic acid are said to be among the end products formed (Cohen, 1920). Nencki (1879) used such a mixture, perhaps unnecessarily, to reinforce the exclusion of air in his constricted tube and plunger device for liquid media; but the first significant application of this principle was by Hans Buchner (1888) who believed that there was little or no evidence that the volatile products formed interfered with bacterial growth under practical conditions. He invented the method of stoppering a small tube of inoculated liquid or solid media inside a larger one containing 1 gram pyrogallic acid crystals and 10 cc. 10 per cent lye per 165 to 175 cc. air space. In the suggestion of Esmarch roll cultures and plate cultures in a closed bell jar, Buchner essentially anticipated the principal lines of development of apparatus subsequently designed for the more efficient utilization of this principle, i.e., methods using tubes and methods using plates.

In addition, we may mention certain methods invented for the observation of bacteria microscopically under anaerobic conditions, as, for example, the hollow-ground slide method of Nikiforoff (1890), the moist chamber methods of Braatz (1890) and Itano and Neill (1921), for the cultivation of anaerobes in hanging drops, and the flattened tube device of Dunham (1897) for

the study of motility under anaerobic conditions, all using alkaline-pyrogallol as a means of reducing the oxygen tension.

PLATING METHODS USING ALKALINE-PYROGALLOL

More important are those modifications which aim to provide conditions such that well-separated surface colonies may be secured as a step in isolation. While Buchner's procedure pertained mainly to the use of tubes, the earliest subsequent development concerned the applicability of the technic to plate cultures, which have been obtained by modification in two general directions, first, by the invention of special plates or combinations of plates to contain the absorbent along with the culture, and second, by the use of bell jars, desiccators, or similar vessels in which ordinary Petri dish cultures might be placed. Both schemes have been utilized with alkaline-pyrogallol alone as the means of reducing oxygen tension or in combination with other methods.

SINGLE PLATING DEVICES

Blücher (1890) described a Petri dish fitted to a saucer with vaselined ground edge, to contain a mixture of lye and pyrogallic acid in the same chamber as the culture, while Gabritschewsky (1891) devised a plate with a moat for the reducing mixture and a ground glass cover with perforations for the passage of inert gas, closure being accomplished by rotating the cover on its vaseline seal, thus throwing the perforations out of coincidence. The principle objection to both plans is that the special plates are not now commercially available, an argument that applies even more to the very complicated and expensive apparatus of Trambusti (1892) and the tubulated Petri dish of Epstein (1900). Turro's (1902) use of the half of a plain Petri dish to hold the alkaline-pyrogallol mixture with a slab of agar carried on a plain glass cover supported on small blocks and luted into position by means of paraffin was a step in the direction of simplicity, but required rapid work in sealing after the mixture was made. The methods of Stüler (1907) and Zinsser (1906), seem to fail in their somewhat extravagant use of reagents and lack of ade-

quacy in the oil seal; my own attempts to cultivate the tetanus
bacillus on the surface of glucose agar by the method of Zinsser
resulted only in failure.   Ogata and Tagenouchi (1914) advocated
a similar method with the addition of a mercury seal, which is
of doubtful value in the form proposed.   The saturation of
cardboard disks with definite amounts of pyrogallic acid for use
in Petri dishes, proposed by Hammerl (1901) and commercialized
by Lentz (1910) requires dishes of unusual depth; the lack of
suitable dishes has prevented a fair test of this method in our
hands.   .

Somewhat similar is the method of Saiki (1909) in which NaOH
solution seeps through a pasteboard box sealed up in a system of
three crystallizing dishes with liquid paraffin and glycerin so
that it saturates the pyrogallic acid after closure.

Heim (1910) and Löwi (1919) placed the alkaline-pyrogallol
mixture in the cover of a Petri dish sealed to the inverted bottom
containing the culture by means of plastiline.

Simonds and Kendall (1912) utilized a vacuum in mixing the
alkali with pyrogallol in a common flat bottle; they also secured
satisfactory surface cultures by the method of Rickards in which
tubes or Erlenmeyer flasks were inverted in a beaker of alkaline-
pyrogallol, a wasteful practice, as contrasted with their own
method and some others.

The method of McLeod (1913) had for its object the sealing of
the air space containing the culture prior to making the mixture
of alkali and pyrogallol, so that all the oxygen absorbed might be
derived with certainty from within the chamber.   This device
comprised a glass plating dish with upcurled edges, to prevent
escape of infected droplets, which after streaking was to be in-
verted over a porcelain capsule partitioned for the receipt, on
one side of pyrogallic acid, on the other strong alkali, through a
central perforation.   The two were then luted together by means
of modelling clay and the mixture was made after the seal was
completed, an altogether ideal arrangement it would seem, since
a large area is provided for absorption.   In England, Henry
(1917) successfully used essentially the same idea in an impro-
vised assembly of sheet iron can tops and Petri dishes, modifica-

tions of which were later described by Wilson and Steer (1918); while in Germany, Brekenfeld (1924) invented a Petri dish over rubber washers on a plate and combined the use of vacuum with that of alkaline-pyrogallol.

COMPOUND PLATING DEVICES (JARS, BELLS)

The Novy (1893) (1894) jar principle already discussed in connection with inert gases was utilized with reducing agents by Arens (1894), who recommended the isolation of *B. tetani*, *B. Chauvoei*, and *B. oedematis-maligni* from Petri dishes in a jar containing sand mixed with dry pyrogallic acid crystals, upon which a dish of 10 per cent lye could be tipped after sealing. Arens dispensed with the use of hydrogen. Zettnow (1894), on the other hand, combined the use of hydrogen with the alkaline-pyrogallol. The use of sodium mercury amalgam with pyrogallic acid and water, with incidental production of hydrogen, by Durham (1896) has been already noted. This method was conveniently operated in a candy jar with iron top and metal pet cocks for evacuation by water pump, in this respect antedating the suggestion of Gage and Spurr (1913). Ayer's (1910) also invented a less fragile type of jar in spun copper with rubber stoppers for use with gases, and alkaline-pyrogallol.

Ucke (1898) appreciating the value of making the mixture of alkali and pyrogallol after closing the apparatus, placed the pyrogallic acid in a small beaker floating in a larger beaker of alkali, running in, under partial vacuum, sufficient water to sink the smaller dish.

Klein (1898) used a simple bell jar, sealed on a plate with beef tallow and wax, for evacuation by water pump through a single rubber stopper and tube which was later sealed by means of a pinch cock, in combination with alkaline-pyrogallol.

Kabrhel (1899), in testing the anaerobiosis of media with methylene blue in an apparatus identical with Klein's, except for the use of a ground glass stopper and cock instead of rubber stopper and pinch cock, suggested leaving the covers off the culture dishes, in order to permit free diffusion of oxygen from the medium. Petri's suggestion (1900) was similar to that of

Klein, except that a doubly perforated rubber stopper in the top of the bell offered an opportunity to pass hydrogen through the air chamber as an additional means of oxygen reduction. Bullock (1900) used a bell jar with sealed-in glass tubes, having ground glass stop cocks, a more expensive, and fragile, but no more efficacious apparatus. Bullock improved the seal by using "*unguentum resinae*" and utilized a partial vacuum to draw the alkaline solution upon the pyrogallic acid crystals after the bell had been filled with gas, either hydrogen or coal gas. Bullock pointed out that, were it not for the intense heat generated by the solution of KOH in water, which might damage the cultures, water might be drawn upon a mixture of dry granulated lye and pyrogallol. Ruzika (1901), using Kabrhel's apparatus, apparently overlooked this objection, in advocating the mixture of solid KOH with a solution of pyrogallol instead of the reverse. Harrison (1902) also used evacuation as a means of emptying an inverted vial of alkali upon the pyrogallic acid crystals, a method recently rediscovered by Ehrenberg (1927).

Fremlin's simple use of a crystallizing dish with ground glass cover first (1903) for a single Petri plate, later (1904) enlarged to accommodate several, was claimed to be successful for surface colonies of *B. tetani*; methylene blue in alkaline solution of sugar or methylated spirit, used as a criterion of anaerobiosis was decolorized, but the alcoholic solution, though more delicate, was not recommended because of its volatility, since it might inhibit bacterial growth.

In view of the relatively well-developed procedures just outlined, the use of a simple bell sealed down with paraffin by Slupski (1901) in his study of the anaerobic growth of the anthrax bacillus and the use of the metal dish in which Mereschowsky (1903) combined the reducing action of hydrogen with alkaline-pyrogallol, and the cumbersome assembly of Lode (1925) seem to involve a retrogression in technic or at least a useless duplication of description. On the other hand, the application of a desiccator to the purposes of the Novy jar by Bordet (1904) and Laing (1904) would seem to be satisfactory. We have already referred to the device of Wilson (1917) as another successful combination of the

hydrogen and reducing agent principle for plating, and this was elaborated by him in 1925 into a porcelain vessel of special design to combine the principles of evacuation, inert gas, and alkaline-pyrogallol for plate cultures.

USE OF TUBES IN THE ALKALINE-PYROGALLOL METHOD

The first work done with reducing agents, i.e., that of Gunning (1877) (1878) (1879), involved the use of tubes, some of which were quite fantastic, but the first really successful device was that of Buchner (1888). It is interesting to note that the original inclusion of a small tube of inoculated medium inside a larger tube containing the alkaline-pyrogallol by Buchner has continued to be one of the most practical methods for the surface cultivation of anaerobes up to the present time. Buchner must have failed, however, to appreciate the necessity of emphasizing the applicability of his procedure to surface cultivation, if we can judge from his illustration, which only shows a deep culture, for which, as we know, special chemical means of anaerobiosis are superflous.

Buchner's combination of tubes led to several modifications, for example, Lubinsky (1894) suggested that the inner tube be supported in a glass stoppered cylinder by means of a perforated cork, and Wright (1900) (1901) proposed that the pyrogallic acid-lye mixture be carried in a cotton plug under the rubber stopper. But, like Buchner, Wright apparently failed to appreciate the unimportance of the method for any but surface cultures, and so pictured a broth culture. Wright's method was simple and effective and required only attention to certain details of technic including the use of solid media, a rubber stopper and seal, and inversion of the tube, to place it in the front rank of many devices for surface cultivation of anaerobes. Crendiropoulo's (1910) combination of the use of hydrogen with Wright's method is superflous. Mellon (1919), who recently pointed out the defects in Wright's original method, offers an alternative procedure in covering the plug with paraffin which is then perforated, treated with alkaline-pyrogallol and capped with rubber.

Whatever the results may be, it seems to involve considerable unnecessary and messy labor.

It seems almost as if the most conspicuous effect of Buchner's simple application of the principle of absorbing oxygen by means of alkaline-pyrogallol to the cultivation of obligate anaerobes, was to stimulate many bacteriologists to invent as complicated devices for this purpose as possible. Curiously such devices are almost always described as "simple"! Thus Omelianski (1902) devised an outer cylinder capped by a glass cover set in a mixture of wax and vaseline, E.F. Smith (see Hunziker, 1902), an inverted U tube, one leg of which held the slanted culture, the other the reducing mixture (used successfully by Nichols and Schmitter (1906) as well as myself), Buchanan (1914), an inset absorption appliance consisting of a small perforated tube carried on the lower end of the rubber stopper to contain the alkaline-pyrogallol solution (not very successful in my hands), Dimond (1915), a liquid paraffin seal between the inverted inner culture tube floating in the alkaline-pyrogallol solution and the open outer tube, and Giltner (1915) and Kollath and Quast (1925), H tubes (of which the prototype was employed by Gunning and his students (1877), with phosphorus as a chemical reducing agent and criterion of anaerobiosis.

Many years ago I used Buchner's method for the isolation of *C. acnes* on slanted oleic acid agar. The tubes were 15 by 2.5 cm. and 12 by 1.5 cm. respectively. Into the larger, about 10 cc. 10 per cent commercial lye solution were pipetted, on top of which a loose plug of absorbent cotton was placed, the tube then being filled with about 1 gram, usually unweighed, of pyrogallic acid crystals. The medium having been inoculated in the small tubes, it was then used to mix the crystals with lye by pushing these and the cotton plug barrier down into the lye solution, at the same time closely stoppering with a previously well-fitted rubber stopper in the large tube. Either end of the small tube may be inserted first, but better results were secured if the cotton plug was left out and the open end itself submerged in the lye pyrogallol solution. When growth occurred, the tube could be rinsed and replugged with a clean cotton plug from a sterile

tube and handled like any aerobic culture.   For the easy removal of the small tube, a vacuum plunger, made by fastening a rubber stopper onto a glass rod and melting out a concave face by means of a hot test tube, was used.

The simplest and most effective means that I have yet found, after testing many schemes and devices for the surface cultivation of obligate anaerobes, is Wright's method, modified by the use of solid slanted media of any kind, a rubber stopper seal, and inversion of the tube to prevent the absorbing solution from running upon the culture.   The tubes are inoculated with anaerobic bacteria in the same manner as with aerobic bacteria; the cotton stopper is cut off with a pair of scissors and pushed into the tube about 2 cm.; the space is filled with crystals of pyrogallic acid; about 2 cc. of 10 per cent lye are poured upon them; a previously fitted rubber stopper is inserted, pushing the lye solution through the crystals into the cotton stopper, and at the same time the tube is inverted.   A large area of absorption is provided by the cotton fibers and, if precaution is observed to have an excess of pyrogallic acid, few failures result.

When I first used the above method, about one-fourth of the cultures failed to grow.   The reason was not understood until Rockwell (1921) (1924), whose studies have shown the apparent need of anaerobes, perhaps all bacteria, for carbon dioxide, proposed to substitute a mixture of NaH_2PO_4 and $NaHCO_3$ for the lye in Wright's method to provide the required CO_2.   I was never very successful in using Rockwell's formula though it seems quite probable that the principle upon which it is based, is sound.   So far as practical results in growing the obligate anaerobes are concerned, I believe that the absorption of carbon dioxide can be prevented by having always an excess of pyrogallic acid; and the greatly improved growth when this precaution is observed justifies this view.

Surface cultures upon which isolated colonies appear may be used for securing pure cultures but exclusive reliance should never be placed in isolation of obligate anaerobes upon surface cultures, because we have as yet no means for measuring the degree of oxygen tension reduction in such cultures and there is

always the possibility that organisms may be present whose particular requirements in reduced oxygen tension may not be met. Any such might, of course, be transferred along with those that have grown, so preventing purification. Deep methods of isolation are therefore preferable (Hall, 1920) but surface cultures are useful in determining hemolytic action on blood agar or liquefaction of proteins such as coagulated serum, and in studying the form of colonies and of the individual organisms.

THE USE OF CHEMICAL REDUCING AGENTS IN CULTURE MEDIA FOR OBLIGATE ANAEROBES

Glucose as a reducing agent

The most important chemical reducing agent used in media for the growth of obligate anaerobes is glucose. Liborius (1886), while not admitting the indispensability of fermentation in anaerobic growth, clearly recognized its stimulating effect. The relation of sugar to anaerobiosis has been well reviewed by Theobald Smith (1895), who recalled the view of Pasteur, Nägeli and Escherich that fermentation is an essential element in anaerobiosis. Kitasato and Weil (1890) pointed out that sugar may serve not only in reduction of oxygen, but in nutrition as well, and Beyerinck in 1893, according to Smith, concluded that some reducing material *must* be present in anaerobic growth. But the experiments of Smith himself show most clearly the value of glucose as a determining factor in the fermentation tube, where no growth of obligate anaerobes could be obtained without it and facultative anaerobes grew only in the open arm of the tube. It must be recognized now, of course, that many anaerobes can be cultivated in the absence of fermentable carbohydrates, providing that more efficacious means of excluding oxygen than those afforded by the fermentation tube be provided, and that fermentation is not an indispensable factor in anaerobiosis. Smith (1895) thoroughly appreciated the deleterious effect of a fermentable sugar in the medium upon the viability of most aerobic and anaerobic bacteria, a point of view re-emphasized by Ucke in 1898, who spoke of glucose as a two-edged sword. But its harmful effects occur only when in excess, and are indirect, that

is, they are due to the acid formed; when there is less than an excess the limiting H^+ ion concentration is not reached and in most cases the culture again becomes neutral or even alkaline through de-aminization of the protein.   It seems clear also that for most of the obligate anaerobes, excepting those that do not ferment sugars, sporulation probably occurs only in sugar-free media, so that not only is the organism exposed in the presence of an excess of fermentable carbohydrates to the adverse circumstance of acidity, but its own usual means of defence may be weakened or lacking.

Other chemical reducing agents

Kitasato and Weil (1890) undertook to find a stronger reducing agent than glucose which would be effective in a sufficiently low concentration not to inhibit growth.   The substances studied fell into two groups, first, those which in alkaline solutions strongly reduce or absorb oxygen, and second, those whose color depends upon the presence or absence of oxygen.   Among the various organic substances tested, sodium formate was found effective as a reducing agent in as low a concentration as 0.1 per cent and non-inhibitive for *B. tetani*, *B. chauvoei*, and malignant oedema bacilli up to 3 per cent.   The nearest approach to this in the first group was in the case of pyrogallol, hydrochinon, and resorcin, which, while effective at 0.1 per cent, are inhibitive at 0.5 per cent.   In the second group, only indigo blue (sodium sulphonate) was tested and found to approximate the last three in the limits set by inhibition.   Sodium formate and indigo blue formerly found considerable favor in the cultivation of anaerobes in deep media (See Hammerl, 1901), but they are not now used at all; it is impossible to secure surface cultures by their aid alone.

Novy (1893) made a considerable study of glucose and other ingredients of culture media in relation to anaerobic cultures. He considered that glucose (1 to 2 per cent), gelatin (2 to 5 per cent) and litmus, all have valuable reducing properties and that an ideal medium should contain all three.   The advantages of litmus were emphasized especially, since it, like indigo, combines

the properties of a reducing agent and an indicator, being decolorized in oxygen-free media.

So far, the successful use of chemical reducing agents had been restricted to organic compounds; the first inorganic substances to be found useful were sulfur compounds. Trenkmann (1898) determined the limits of concentration within which the bacilli of malignant oedema, tetanus and blackleg would grow in broth containing Na_2S but the results are expressed in drops and therefore have no quantitative value. Hammerl (1901) compared the efficiency of Na_2S, K_2S and NH_4SH as reducing agents, judging the results not alone by the growth of anaerobic organisms, but also by the decolorization of methylene blue. NH_4SH was found to be the most efficacious, from both standpoints. Its instability is a serious bar to its practical use, however. A further contribution was made by Rivas (1902) to the subject of cultivating anaerobes in the presence of Na_2S and NH_4SH, the only tangible result of which was to show the futility of attempting to secure surface cultures by these means.

The reducing action of compounds containing sulfur has recently been studied anew by Hosoya (1925) and others. Hosoya found that the addition of 0.001 per cent l-cysteine hydrochloride greatly stimulated the growth of various obligately anaerobic bacteria in liquid media (pH 7.2 to 7.4) without seals other than depth and that neither l-cystine, taurine, nor thioglycollic acid could replace the l-cysteine for this purpose. In a further report, Hosoya and Kishino (1925) observed that *B. tetani* and *B. botulinus* failed to grow in a gelatin digest medium except when cysteine, sulfuretted hydrogen, cystine, or sodium sulfide (Na_2S) were added. Cysteine and sulfuretted hydrogen were the best and neither could be replaced by l-tryptophane, l-tyrosine, sodium sulfate (Na_2SO_4), acid sodium sulfate ($NaHSO_4$), sodium sulfite (Na_2SO_3), sodium thiosulfate ($Na_2S_2O_3$), taurine, or sodium taurocholate. The results with l-cysteine were duplicated for *B. sporogenes* by Quastel and Stephenson (1926) who attribute the effect to the reducing action of the SH group since thioglycollic acid (compare Hosoya) and glutathione behaved similarly. Quastel and Stephenson's findings with *B. sporogenes* were con-

firmed by Aubertin, Aubel, and Genevois (1928) and extended to *B. putrificus, B. fallax, B. perfringens (B. Welchii); B. bifermentans, B. oedematiens (B. Novyi),* and *B. tetani.* Interesting as they are, the results must be regarded for the present as primarily of only theoretical importance, since they have not made it possible to secure strictly aerobic, i.e. surface growth on solid media of any obligate anaerobe. Guillemard (1911) utilized iron sulfate (0.3 per cent) as a means of reduction. But the medium must be boiled out and growth cannot be secured on the surface of solid media. However, Guillemard found that six parts of iron per 100,000 profoundly affected the metabolism of certain anaerobic bacteria, and Scott (1926) observed that the addition of ferric sulfate to culture media aided *B. Chauvoei* to preserve its virulence. The reducing properties of iron and some of its compounds suggest that it may prove to have special values in anaerobic technic which still remain to be discovered.

Our knowledge of chemical reducing agents may be briefly related to certain biological theories of anaerobiosis which we have already reviewed in another place (Hall, 1928), for the ultimate explanation of aerobic-anaerobic symbiosis and the successful use of animal and plant tissues in the so called "aerobic culture" of obligate anaerobes, must rest in the last analysis upon a true understanding of their physical and chemical bases.

In 1922, M'Leod and Gordon (1922) discovered that the early death of the pneumococcus in cultures was apparently due to the accumulation of peroxide (of hydrogen?), for the production of which evidence was found in the green discoloration of "chocolate" blood agar, and in the liberation of oxygen from pneumococcus cultures by liver catalase. Streptococci and other bacteria also produce peroxide and it was suggested that the well known beneficial action of fresh tissues upon various bacteria is due to the destruction of detrimental peroxide by catalase.

That many bacteria also produce catalase has been known since the work of Gottstein (1893) and Beijerinck (1893). The latter also pointed out that the lactic acid bacteria do not produce it, and Lowenstein (1903) showed that the anaerobic bacteria resemble the lactic acid bacteria in this respect.

Callow (1923) suggested that, if the obligate anaerobes lack catalase, their inability to grow in the presence of oxygen might be explained by the toxic action of peroxide. In the absence of air, no peroxide would be formed and growth would occur. She showed that while nine aerobes produced abundant catalase, seven anaerobes and three streptococci produced only the slightest possible traces of this enzyme. Yet it was impossible to detect peroxide in anaerobic cultures exposed to the air; neither could the anaerobes be cultivated upon the surface of solid media containing catalase in contact with air. These results were confirmed by M'Leod and Gordon (1923) and supported by the information that *B. tetani, Vibrion septique,* and *B. Welchii* are highly susceptible to slight traces of added peroxide of hydrogen. As these investigators have suggested, their failure to demonstrate peroxide formation by anaerobic cultures exposed to the air does not necessarily invalidate their theory, since the amount of peroxide required to inhibit the growth of anaerobes is apparently much less than that necessary for its chemical demonstration. While they were unable to secure any evidence that liver catalase favors the growth of *B. tetani,* good growth of *B. Welchii* was secured with catalase in oxygen tensions that tended to inhibit without it. They further found (1925) that there is a close parallelism between the reduction of glutathione, the dissolving of cystine, and the discoloration of chocolate blood agar and pointed out that all these phenomena can be explained by the assumption that the bacteria responsible for them generate active hydrogen.

Avery and Morgan (1923) (1924) were also able to cultivate certain obligately anaerobic bacteria in broth containing unheated plant tissues, for a few transfers. But surface cultures in air failed, as usual, even with *B. histolyticus,* which is really not an obligate anaerobe but can be cultivated aerobically for an apparently indefinite number of generations (Hall, 1923) (Hall and Whitehead, 1927). Perhaps the most important discovery of Avery and Morgan, was that the formation of peroxide by the pneumococcus is favored by free access of air and absence of catalase, while anaerobic conditions, or the presence of catalase

from plant tissues, inhibit the formation and accumulation of peroxide. But, like the English investigators, they were unable to demonstrate the formation of peroxides by the obligate anaerobes.

The first direct demonstration of peroxide formation by an obligate anaerobe was accomplished by Hagan (1924) with *Actinomyces necrophorus*, young cultures of which exposed to the air give the benzidine test for peroxide of hydrogen. But M'Leod and Gordon (1925) were unable to demonstrate peroxide in cultures of *B. tetani* and *B. Welchii* by Hagan's methods and the catalase-peroxide theory remains, for all obligate anaerobes except *A. necrophorus*, merely an interesting hypothesis supported only by indirect evidence. Yet Neill (1925) holds that the oxidation and reduction reactions of sterile plant tissues are essentially identical with those of pneumococci and anaerobic bacteria, and furnish the best explanation, thus far, for the beneficial action of *raw* plant and animal tissues upon anaerobic growth. On the other hand Novy (1925) holds that "the inability of anaerobes to grow in the air is not due to the hypothetical production of peroxide and to the absence of catalase" but "that the fundamental difference between obligative aerobes and anaerobes lies in the nature of the respiratory enzymes, which are designated as aerase and anaerase, respectively. The potato and the facultative anaerobes possess both types; that present in obligative anaerobes can function only in the absence of oxygen, while that of the aerobe can work only in the presence of oxygen." All living protoplasm respires and Novy's conception of the beneficial action of plant tissues upon anaerobic growth dispenses with the peroxide-catalase hypothesis and emphasizes the idea that obligate anaerobes grow in the presence of raw plant tissues because the latter reduce the oxygen tension, just as aerobic bacteria do, below the toxic concentration.

Useful as chemical reducing agents are in any culture medium for the growth of obligately anaerobic bacteria, we are forced to conclude that, as yet, none has been found that permits these organisms really to grow as aerobes grow, that is, upon the surface of a solid medium exposed to the atmosphere through a

cotton plug. As Hunziker said in 1902, "no matter how great the reducing power of any one of these chemical reducing agents may be, they are not able to make harmless the atmospheric oxygen, which re-enters the medium when the latter is poured into Petri dishes. . . . . In order to use reducing agents successfully, they should be used in connection with some other method for cultivating anaerobic bacteria."

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THIRTY STRAINS OF GRAM-POSITIVE COCCI ISO-LATED FROM CASES OF GENITO-URINARY INFECTIONS

ASYA M. S. STADNICHENKO

Research Laboratory, Municipal Tuberculosis Sanitarium, Chicago, Ill.

Thirty strains of Gram-positive cocci were isolated from 30 cases of genito-urinary infections. All the specimens were collected by the method routinely employed in the Brady Urological Institute,[1] that is, the urinary meatus was cleansed with alcohol, and the anterior urethra by injection of 1:500 per cent Meroxyl. The first urine was allowed to escape, and a specimen was then obtained in a sterile glass tube, the cotton stopper of which was wrapped in paper. In the laboratory specimens were transferred into sterile centrifuge tubes by means of sterile pipettes, and were centrifuged for three to five minutes at 2200 revolutions a minute. After decanting the supernatant urine, direct smears were made and the sediment cultured.

Since coccal urethral contamination is a frequent occurrence, great care was taken not to include in this series any organisms of such origin. All the strains were isolated from cases in which direct smears showed both Gram-positive cocci and leukocytes. Also, the bacteriological findings were correlated with the clinical histories,[2] and no case of known possible contamination was included.

All the strains came from cases of a single infection (Gram-positive cocci only), except two cases, where direct smears showed

[1] The experiments serving as the basis of this paper were carried out by the author while working in the Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Maryland.

[2] It is a pleasant duty to acknowledge my great indebtedness to Dr. J. A. C. Colston with whose help it was possible to make these correlations. Also I wish to express my gratitude to Justina H. Hill for her interest in this work.

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both Gram-positive cocci and Gram-negative bacilli. In these two instances special care was taken to isolate the cocci in pure cultures. Later, one of these cases proved to be a fatal case of *Staphylococcus aureus* septicemia. Staphylococci isolated from the patient's blood were found to be identical with the organism that came from his bladder.

In most cases organisms were isolated from the bladder only, but in cases where the kidneys were involved also, and a catheterized specimen obtained, a comparative study was made. Invariably, organisms from the same host gave the same reactions in all the tests employed.

All the strains were isolated by fishing a single colony from twenty-four lactose-agar plates. Sub-culturing of the stock cultures was done every five or six weeks. Some of the cultures were in my possession for over two years, during which period they were cultured and re-cultured scores of times. At the end I was not able to find any variation in their cultural characteristics, except one case of the liquefaction of gelatin, which will be discussed later.

GRAM-STAIN

Nicolle's one-minute stain was used:

$\begin{cases} \text{1 cc. saturated alcoholic solution of gentian violet.} \\ \text{9 cc. 2.5 per cent aqueous solution of phenol.} \end{cases}$
Stain 20 seconds and pour off.
Stain Gram's iodine 20 seconds.
Decolorize with solution 3 parts of alcohol and 1 part of acetone.
Counterstain with Carbol-fuchsin 1 part and 19 parts of H_2O.

The organisms were stained at different times. Invariably, all of them up to a month old retained gentian violet; but with greater age they retained gentian violet only in spots. Such cultures when transferred to a new medium, always re-gained their ability to retain the stain.

CHROMOGENESIS

Chromgenesis was determined by the method described by Winslow and Winslow (1908). Cultures were grown on agar

slants for fourteen days at room temperature (20°C.), and in the incubator (37°C.). Matching was done against the Winslow color chart. Fourteen strains were matched against different hues of orange, one strain gave a typical citron color, and fifteen were white. All the strains showed pigment production within eighteen to thirty-six hours. No new appearance of pigment was observed after thirty-six hours, but in many instances the pigment became more pronounced with age. It seems that the pigment production was better at room temperature than in the incubator, but the difference was not very marked.

All the strains were also grown on Loeffler's serum and potato media. The pigment production on Loeffler's serum seemed more intense than on agar, and this difference was especially noticeable in the first eighteen to forty hours. Potato medium in most cases confirmed the observations made on agar and Loeffler's serum media. One strain, however, while growing well on Loeffler's and agar, failed to grow on potato, in spite of the fact that several transfers were made. It was a white coccus that did not liquefy gelatin, or reduce nitrates; it produced acid in glucose, lactose, sucrose, maltose and mannitol; it decomposed urea, and was classified as *M. ureae*. Two other strains of the same species in this series produced a good growth on potato medium.

GELATIN

Gelatin liquefaction was always tested with a freshly isolated organism. In the course of study this test was repeated on some of the strains two and three times, and, finally, just before the work was completed it was repeated on the whole series. On all the repeated tests, all the organisms gave the same reaction as when first isolated. There was one exception to this, however; one strain (*S. albus*), that was in my possession for two years, at first liquefied gelatin, but later lost that power, though all of its other cultural characteristics remained constant. It was possible later to re-isolate this coccus from the patient's urine. This freshly isolated organism liquefied gelatin, as before. Therefore this particular strain conforms to Kruse's (1896) observation,

that organisms may lose their ability to liquefy gelatin when growing on artificial media for some time. However, the fact that only one strain in this series lost its ability to liquefy gelatin, while a few others that were in my possession as long continued to liquefy it, suggests that different organisms are affected differently by growth on artificial media.

Organisms were grown in gelatin for thirty days at room temperature (20°C.) and in the incubator (37°C.). At the end of this period the set that was growing in the incubator was put into the ice box for one hour. No difference was observed between these two sets. The percentage of liquefiers was higher in the orange group, 9 strains (64 per cent) out of 14 of these liquefying gelatin, while in the white group 6 strains (40 per cent) out of 15 possessed this power. One strain of citron coccus also liquefied gelatin.

MILK

Organisms were grown in litmus milk for fourteen days at 37°C. The orange cocci exhibited less variation in their reactions than the white ones, but the only strain that gave an alkaline reaction in this series belonged to the orange group.

Table 1 gives reactions in litmus milk.

CARBOHYDRATES

The following carbohydrates were used in this series: glucose, sucrose, lactose, maltose, salicin, mannitol, glycerol, dulcitol, dextrin, raffinose and inulin. The medium was a beef-infusion agar made sugar-free, containing 1 per cent of the carbohydrate and double indicators (brom-cresol purple with cresol-red); the reaction was adjusted carefully in accordance with the procedure prescribed in the Manual of Pure Culture Study (1924). Since the double indicators proved to be very sensitive in registering even a slight change from a neutral point, no attempt was made to read pH value. All the strains in this series which fermented any carbohydrates fermented them so strongly that there was no doubt as to the nature of the reaction.

Inoculation were made both along the slant and deep into the

butt.   Cultures were always incubated for ten days before being discarded as negative.   However, all the carbohydrates that were fermented in this series were fermented within three days.   There was only acid; no gas production.

Table 2 gives reactions with the carbohydrates studied.

TABLE 1

Action on milk

MILK	NUMBER	ORANGE	CITRON	WHITE
No change............................	1	0	0	1
Acidity: Not sufficient for coagulation...	4	2	0	2
Acidity: Sufficient for coagulation.........	22	11	1	10
Peptonization............................	2	0	0	2
Alkalinity...............................	1	1	0	0
Total.................................	30	14	1	15

TABLE 2

Fermentation of carbohydrates

CARBOHYDRATES	NUMBER	ACID	ORANGE	CITRON	WHITE
Glucose........................	30	30	14	1	15
Sucrose........................	30	30	14	1	15
Maltose........................	30	28	14	1	13
Lactose........................	30	28	13	1	14
Mannitol.......................	30	27	14	1	12
Glycerol........................	30	26	14	0	12
Salicin.........................	30	9	2	0	7
Dextrin........................	30	3	0	0	3
Dulcitol........................	30	2	0	0	2
Raffinose.......................	30	2	0	0	2
Inulin..........................	30	1	0	0	1

Winslow, Rothberg and Parsons (1920) observed that the strains isolated from pathogenic conditions have a marked tendency to ferment carbohydrates rather strongly.   Strains in my series exhibited the same tendency.   As table 2 shows, all the strains (100 per cent) fermented glucose and sucrose, and most of them (94 per cent) fermented maltose and lactose; mannitol (90 per cent); and glycerol (87 per cent).   The fermentation of mannitol in this series accords with Dudgeon's (1908) observa-

tion, that mannitol is acidified by most of staphylococci obtained from pathogenic sources. Salicin was fermented by 9 strains only, and of these 9 strains 7 were white cocci. Dextrin, dulcitol, raffinose and inulin were fermented by only a few white strains,— none of the orange strains fermented the above mentioned carbohydrates. In the fermentation of carbohydrates, as in milk, the white strains exhibited more variation in their reactions than the orange strains.

UREA

Pasteur (1860) was the first to notice a "spontaneous" fermentation of urine. In (1862) he had proved that this fermentation of urine was produced by a white micrococcus, which was capable of breaking down urea to ammonium carbonate. Miquel (1888) in his laboratory had grown *urococcus* (as he named the coccus capable of fermenting urea), and studied carefully its property in regard to urea fermentation. An organism, from decomposing urine, was described by Flügge (1896), as a white micrococcus generally found in the human or animal body. Flügge recorded that his organisms differs from Pasteur's in its ability to liquefy gelatin, and proposed to name it *M. ureae-liquefaciens*. Hucker (1924) in describing *M. ureae* states that it is a primary soil form, the fundamental characteristic of which is ability to utilize urea.

In my series all the strains have been grown in plain broth, containing 2 per cent urea and phenolphthalein as an indicator. The medium was adjusted to acidity. With the decomposition of urea and the production of alkalinity, there developed a pink color and a strong ammoniacal odor. Cultures were incubated for ten days before being discarded as negative. The strains in this series that were capable of decomposing urea did so within eighteen to forty-eight hours.

In this series, 14 strains decomposed urea; 8 of them were orange strains and 6, white. It appears, therefore, that there is no correlation between pigmentation and urea utilization; nor do any cultural characteristics appear to be common to all these 14 strains, save fermentation of glucose and sucrose. Most (10) of the urea-decomposing strains produced sufficient acid to curdle

milk.   The high percentage of urea-positive strains in this series may have some connection with the source of the specimens (urine), but the data in hand are not sufficient to justify a positive statement.

INDOL

Bayne-Jones and Zinninger (1921) gave us, perhaps, the best survey of the literature on the subject of indol production by staphylococci.   Their own intensive work in this line confirms the findings of most investigators, viz., they were not able to demonstrate indol-positive staphylococci.   In my series all the strains were cultured in 1 per cent peptone and 0.5 per cent K_2HPO_4.   Tests for indol production were made by the para-dimethyl-amido-benzald-benzaldehyde method after the second, fourth, and seventh day of incubation at 37°C.   All the cultures were indol-negative.

NITRATES

Cultures were grown in nitrate broth, and nitrate reduction was tested with sulphanilic acid and a-naphthalamine after the second, fourth, and seventh day of incubation at 37°C.   The orange cocci gave a higher percentage of nitrate-reducing strains than the white cocci.   Nine (64 per cent) out of 14 orange strains reduced nitrates, while in the white group only 8 (55 per cent) out of 15 possessed this power.   The only citron strain in this series also reduced nitrates.

The length of incubation proved of no significance in this test. There was no difference in results of these three sets.

HEMOLYSIN

Neisser and Wechsberg (1901) and Kutscher and Konrich (1904) showed that staphylococci (*S. albus* and *S. aureus*) are capable of producing hemolysin, but they were not able to find a purely saprophytic form with this characteristic.   On the other hand Julianelle (1922) found that all of his strains, both parasitic and saprophytic, were capable of producing hemolysin.   Hucker (1924) found only a certain percentage of hemolysin-positive strains in his large series.   From his work it appeared that the

white strains gave a larger percentage of hemolysin-positive strains than any other group of cocci (orange or yellow; he found none among red cocci). Mellon and Caldwell (1926) reported most of their strains hemolysin-positive.

In this series Brown's (1919) method for making blood plates was employed. Reading was done after twenty-four and forty-eight hours; no further observation was made. Out of 30 strains 6 gave positive reactions; 5 of them were white strains, and only one orange. Hemolysin in all 6 cases was of the *beta* type. There were no other cultural characteristics in common (save fermentation of glucose and sucrose), nor could any special importance be attached to these six strains from a clinical point of view.

CAPSULES

Strains were grown on milk-agar for eighteen hours, and then were examined with India-ink for capsule formation. No definite capsules were observed in any of these strains.

MOTILITY

Strains were grown in peptone broth and, after twelve, eighteen and twenty-four hours of incubation at 37°C., were examined for motility. A little more than a self-rotation movement was observed in some of the cultures, but none exhibited a typical rapid movement across the stage. The length of incubation made no difference.

CLASSIFICATION OF THE STRAINS

The bacteriological findings resulting from the foregoing experiments served as basis in classifying strains in this series. I insert specific names already in use. Work of different authors including Migula (1900); Chester (1901); Winslow and Rogers (1905–1906); Winslow and Winslow (1908); Kligler (1913); the report on the Committee on Classification (1917); Buchanan (1917); Winslow, Rothberg and Parsons (1920); Hucker (1924), and Bergey (1925) were used as guides in selecting specific names for my strains.

In the orange group there were 9 strains that were gelatin and

lactose positive; all of them produced sufficient acid to curdle milk, and most of them (7) also reduced nitrates.   The name *Staphylococcus aureus* was applied to them, but to find a proper name for the strains in this group that were gelatin-negative, but lactose-positive is difficult.   Winslow, Rothberg and Parsons (1920) found only a few strains with such characteristics and suggested leaving them nameless at that time.   They, like most investigators, applied the name *Staphylococcus aurantiacus* to an orange strain that was gelatin and lactose negative.   However, Hucker (1924) in his work finds that *M. aurantiacus* is closely related to *M. aureus*, but differs in its ability to liquefy gelatin, and also differs serologically.   Although serological tests were not employed in this work, the name *M. aurantiacus* was applied to 4 strains that were gelatin-negative, but lactose-positive; also to one strain that was gelatin- and lactose-negative.   Of these 5 strains 2 only reduced nitrates.   The only strain of *Staphylococcus citreus* that I have in this series liquefied gelatin; reduced nitrates, and produced sufficient acid to curdle milk.

In the white group there were 3 strains of *Staphylococcus albus,* all of which fermented lactose; liquefied gelatin; reduced nitrates, and produced sufficient acid to curdle milk.   There were 2 more strains with the same characteristics, except that both of them peptonized milk rather rapidly.   The name *M. caseolyticus*, proposed by Evans (1916), was applied to these two strains. Three strains were identified as *M. ureae*; all of them decomposed urea, produced sufficient acid to curdle milk, but failed to reduce nitrates, or liquefy gelatin.   There was one strain that liquefied gelatin and decomposed urea, but did not reduce nitrates, nor did it ferment lactose or mannitol; litmus milk was unchanged. The name *M. ureae liquefaciens* (Flügge) was applied to it.   Three strains were classified as *Staphylococcus epidermidis* (Kligler). They did not liquefy gelatin, but reduced nitrates; all of them fermented lactose, mannitol, maltose and glycerol; two of them fermented salicin.   Two strains were classified as *M. candidus.* They did not liquefy gelatin, nor did they reduce nitrates.   One strain was classified as *M. tetragenus* on account of its typical cell-arrangement.

These 30 strains were isolated in connection with the following infections: prostatitis; urethritis; pyelonephritis; urinary calculus; ulcerated cystitis; epididymitis and prostatic abscesses. It was found that, in most cases, orange strains produced more severe infections than white, although certain strains in the white group are among the most virulent in the whole series.

SUMMARY AND CONCLUSIONS

Thirty strains of Gram-positive cocci isolated from thirty cases of genito-urinary infection were diagnostically studied. Following are the principal results:

1. The most prevalent type of infection in this series is prostatitis. However, no correlation is yet established between this type of infection and the strain of cocci isolated in connection with it.

2. Strains in this series have a tendency to ferment carbohydrates strongly. The following carbohydrates were fermented by almost all the strains: glucose, sucrose, maltose, mannitol and glycerol.

3. The ability of strains in this series to decompose urea was very pronounced.

4. On the whole, the white strains exhibited more variation in their cultural characteristics than the orange.

5. The orange group proved to have a higher percentage of gelatin-liquefying; nitrate-reducing; milk-coagulating, and urea-decomposing strains. In general, the orange strains were more active, and the infections, in connection with which they were isolated, were more severe.

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THE EFFECT OF FILTRATES OF CERTAIN INTESTINAL MICROBES UPON BACTERIAL GROWTH

MARGARET F. UPTON

Department of Bacteriology and Public Health Washington University School of Medicine

INTRODUCTION

In undertaking an investigation of the intestinal flora of normal breast-fed and artificially fed infants (work about to be published), the writer was impressed with the apparently great inhibitory action which *Bacillus bifidus* exerts upon the development of other intestinal bacteria in laboratory cultures. If one prepares two inoculated lactose-liver-agar plates, using the spiral streak method (Varney, 1927), from undiluted intestinal material from a healthy breast-fed infant a week or more old, and incubates one plate aerobically and the other anaerobically, the resulting cultures are strikingly different. As has been pointed out by Tissier and Dreyfus (1925) and by other authors, in the intestinal flora of such infants only three types of bacteria are constantly present: *Bacillus bifidus*, *Bacillus coli*, and the enterococcus or *Micrococcus ovalis*. As *B. bifidus* is an anaerobe, no colonies of this type develop upon the aerobic plate. Consequently this culture shows a relatively even distribution of *B. coli* and *M. ovalis*. On the anaerobic plate, however, enormous numbers of small colonies of *B. bifidus* appear and only occasional colonies of *B. coli* and *M. ovalis*. The proportions of the different colonies are closely comparable to those of bacteria observed on the Gram-stained direct smear from fecal material where one encounters *B. bifidus* in almost pure state. As there are doubtless approximately the same number of *B. coli* and *M. ovalis* organisms in the two bits of material streaked, there is evidently an inhibition of growth due to the presence of *B. bifidus* when conditions favor-

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able to its development are present. *B. coli* and *M. ovalis* grow anaerobically as well as under aerobic conditions. Similar plates made from feces from a bottle-fed child differ very little from each other with respect to the development of these two bacteria. The anaerobic plate shows *B. bifidus* but usually it occurs in such small numbers that its presence has no appreciable effect upon the other types.

This inhibitory effect of *B. bifidus* upon other bacteria was investigated by Kling (1914). He concluded from his work that the bactericidal action exerted was due entirely to the acid-producing properties of the bacterium, since, when cultures were neutralized, no inhibition of other forms took place. He made no distinction between "acid-producing properties" in terms of hydrogen ions and the molecular character of the acids involved. As was found in experiments about to be described, neutralizing the bacterial culture (or filtrate) will indeed destroy the bactericidal property but it appears that when the hydrogen-ion concentration is only slightly lowered with alkali, factors other than the pH become apparent which tend to discourage bacterial growth.

EXPERIMENTAL

The three organisms used in this study were *B. coli* (fermenting sucrose), *M. ovalis* and *B. bifidus.* They were freshly isolated strains from the stools of normal infants. The same strains were used throughout, except that in the work with the organic acids, and in one experiment with the filtrates, different strains of *B. coli* and *M. ovalis* were used for inoculating purposes.

The medium for these experiments was selected with special reference to the requirements of *B. bifidus*, as that organism is the most difficult of the three to cultivate. As a result of some experimenting it was found that after isolation on lactose-liver agar the organism could be transferred to lactose-peptone broth. A broth containing Difco proteose peptone (0.5 per cent) and dipotassium phosphate (0.5 per cent) was adjusted to pH 7.0 with sulfuric acid. A 20-per cent solution of lactose in distilled water was sterilized by passage through a Berkefeld filter to avoid

any possible hydrolysis by heating. The lactose was then added to the sterile broth so that there was a final concentration of 1 per cent. This lactose medium was used in all the experiments described in this paper. Each of the three organisms was inoculated into flasks. *B. coli* and *M. ovalis* were grown aerobically at 37°C. for from three to five days. It was found that the pH of the cultures remained the same after three days' incubation. *B. bifidus* was incubated in an anaerobic phosphorous jar for 4 days or until growth appeared heavy. At the end of the incubation period only *B. coli* appeared to be still viable. The broth containing the organisms was then centrifuged and passed through a sterile Berkefeld filter. The pH of the *B. coli* filtrate was 5.0 to 5.3, of the *M. ovalis*, 4.8 to 4.9, and of the *B. bifidus*, about 4.0. After some preliminary work it was decided to run experiments at three representative hydrogen-ion concentrations. Accordingly, flasks of each filtrate were adjusted by sodium hydroxide or hydrochloric acid to pH 4.5 pH 5.0 and pH 6.0. Gillespie's standards of pH values, using methyl red as indicator (Clark, 1922), were employed in estimating pH. The pH value 4.5 was selected because with this acidity, *B. coli* is still able to develop, although inhibited somewhat by the high hydrogen-ion concentration. The "critical zone" for *B. coli* is described by Cohen and Clark (1919) as lying between pH 4.6 and 5.0 in media adjusted with hydrochloric acid. The pH value 5.0 is approximately the hydrogen-ion concentration of the breast-fed infant's feces and pH 6.0, that of the artificially fed infant's. In fact, in experiments where results were estimated at pH 7.0, there was practically no difference between pH 6.0 and pH 7.0 so that neutralizing effects are apparent at the lower pH value. Control flasks, using uninoculated broth, were adjusted in the same way. Four sets of sterile test tubes, containing 5 cc. each of filtrate at each pH, were set up. Three sets were inoculated with equal amounts (usually 0.2 cc.), each, of a suspension of one of the three organisms from a slant washed down with broth. The fourth set was left uninoculated as a control. At first the sets containing *B. coli* and *M. ovalis* were incubated aerobically, but it was found that the organisms remained viable longer under

anaerobic conditions and there was no loss in volume by evaporation. Consequently the sets were incubated in the anaerobic jar.

After twenty-four hours the turbidity in each tube was recorded, as indicated in table 1. It is quite evident that *B. coli* and *M. ovalis* do not produce the same degree of turbidity. The expression "++++" therefore indicates a maximum turbidity for the organism under consideration and the other symbols refer to the turbidity, relative to this maximum. Every day, beginning with the first after inoculation, one loopful of culture from each tube was streaked on a portion of lactose agar plate. This transplanting was done until no colonies grew from the tubes. These transplants gave a good idea of the viability of the

TABLE 1

Growth of B. coli and M.ovalis in filtrates and broth as shown by turbidity in twenty-four hours

MEDIUM	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
B. coli filtrate...............	Clear	±	++++	Clear	Clear	++++
B. bifidus filtrate...........	Clear	±	+++	Clear	Clear	+++
M. ovalis filtrate...........	Clear	+	+++	Clear	Clear	++
Lactose broth...............	++	++	++++	±	±	++++

organisms. Organisms may remain alive for many days in a culture which shows practically no turbidity. One often sees experiments recorded in the literature where the clarity of a tube is taken as an indication of sterility, whereas, often, the organism may still be viable without visible growth. The amount of growth gave a good indication of the relative numbers of living bacteria in the tubes and an approaching dying-out of the culture could be predicted by a falling-off in the number of colonies.

Similar tests, using *B. bifidus*, were attempted but owing to the peculiarities of this organism difficulties were encountered which made the meager results obtained unreliable for comparative purposes. Usually, the organism failed to grow, or, if growth took place, it died out so quickly in the tubes that transplants for

purposes of testing viability were unsatisfactory. Probably the small volume of culture used tended to restrict growth and what growth took place was not sufficient to be detected by the rather rough methods employed.

Table 1 shows, by the turbidity produced in twenty-four hours, the effect of the filtrates upon growth of the bacteria. As may be seen by comparing the filtrates at pH 4.5 and 5.0 with the corresponding tubes of lactose broth, bacterial growth is definitely inhibited in the filtrates, whereas in broth it is allowed to proceed. At pH 6.0, or, as the reaction approaches neutrality, growth in the filtrates is little less marked than in the broth. Table 2 corroborates these results by showing that bacteria remain alive longer in broth than in the filtrates at pH 4.5. It, moreover, indicates

TABLE 2

Viability of B. coli and M. ovalis in filtrates and broth as shown by number of days the organisms remained alive

MEDIUM	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
B. coli filtrate	0	1	9	0	5	9
B. bifidus filtrate	0	1	5	0	9	9
M. ovalis filtrate	1	2	6	2	9	9
Lactose broth	4	5	4	7	5	5

that, as the filtrates approach neutrality, they may even have a more beneficial effect upon the bacteria than broth alone. *M. ovalis* is less inhibited than *B. coli* at pH 5.0, as shown in table 2. The fact that the organism remains viable for a long time at this pH whereas the broth shows little evidence of cell multiplication, indicates that the effect is inhibitive rather than bactericidal.

This brings us to the question of what metabolic products yielded by the bacteria may be the means of bringing about such results, for it is quite evident that the hydrogen-ion concentration alone cannot in these experiments account for the inhibition. In the presence of fairly large amounts of carbohydrates, it has been long known that bacteria produce but slight, if any, nitrogenous changes in the substrate. The buffer used, only 0.5

per cent, would not be sufficient to allow even the nitrogenous changes described by Slanetz and Rettger (1928). The carbohydrate was not used up in these cultures, as was proved by a positive Benedict's sugar test on the filtrates at the end of the incubation period. Moreover the aciduric organisms, *M. ovalis* and *B. bifidus*, have been shown by Kendall (1921) to have practically no ability to alter nitrogenous substances. The acidproducing properties, of the three organisms studied are, however, well known and it was decided to determine roughly the proportions of volatile and lactic acids produced from lactose sugar by these organisms.

METHOD FOR DETERMINING VOLATILE ACIDS

The method of precipitating the proteins was a modification of a method for determining the volatile acids present in adult feces developed by W. H. Olmsted in work to be published. To 250 cc. of a five- or six-day culture were added 150 cc. of 10 per cent mercuric chloride (in 4 per cent HCl). A thick suspension of calcium hydroxide was added until the solution was decidedly alkaline, and the volume was made up to 500 cc. After centrifuging, 200 cc. of the supernatant fluid, made acid with sulfuric acid, were treated with hydrogen sulfide, aerated, filtered and diluted to 250 cc.

To 100 cc. of filtrate were added 70 grams magnesium sulfate and 2 cc. of 50 per cent sulfuric acid. The mixture was steam-distilled at the rate of 100 cc. in nine to ten minutes. The 100-cc. fractions were titrated with tenth-normal and one-hundredth-normal sodium hydroxide. A filtrate from a blank of uninoculated broth was prepared and distilled in the same way, and the readings deducted from the readings on the cultures for the final result.

METHOD FOR DETERMINING LACTIC ACID

Mercuric nitrate solution was used in precipitating the protein. To 50 cc. of broth or culture were added 5 cc. of mercuric nitrate solution (in 1 liter, 160 cc. of concentrated HNO_3 220 grams HgO, 60 cc. of 5 per cent NaOH); 30 per cent sodium hydroxide was

added until a yellow precipitate appeared but not enough to cause the solution to become alkaline. After making up to 100 cc., barium carbonate was added until the solution was neutral to litmus. After filtering, sodium sulfate was added to precipitate the barium sulfate. The solution was then made acid with concentrated sulfuric acid and treated with hydrogen sulfide to remove the mercury; the solution was then aerated, a drop of 10 per cent copper sulfate solution added and the solution filtered. To each 10 cc. of filtrate, was added 1 cc. of 20 per cent copper sulfate and enough calcium hydroxide to make it alkaline. This removed the sugar. Five cubic centimeters of the filtrate were

TABLE 3

Volatile and lactic acids recovered from six-day anaerobic cultures in 1 per cent lactose-buffered-peptone broth, expressed in terms of 0.1 N NaOH per 100 cc. culture

ORGANISM	TITRATED ACIDITY	VOLATILE ACIDS		LACTIC ACID		PER CENT OF TITRATED ACIDS RECOVERED
		Total	Per cent of titrated acids	Total	Per cent of titrated acids	
B. coli....................	26.5	12.4	46.8	2.9	10.9	57.7
M. ovalis.................	25.3	3.7	14.6	16.7	66.2	80.8
B. bifidus...............	100.2	64.6	64.6	22.6	22.6	87.2

used for the lactic acid determination. The method and apparatus used was that described by Friedemann, Cotonio and Shaffer (1927). A blank was run on a filtrate from uninoculated broth and the readings deducted. One-hundredth-normal iodine was used for titrating.

The results obtained with a representative strain of each type are noted in table 3. While actual figures varied somewhat on the several different cultures, the results are consistent. One can readily see that the three organisms differ markedly with regard to the utilization of the carbohydrate. *B. bifidus* produces the largest titratable acidity, *B. coli* and *M. ovalis* about equal amounts. *M. ovalis* produces less lactic acid than does *B. bifidus*, but this acid forms a greater percentage of the total acid production. *B. bifidus* produces large amounts of volatile

acids both in actual amount and in proportion to the titrated acidity. *B. coli* produces more volatile acids than *M. ovalis* but much less lactic acid. Comparing the distillation curves of the volatile acids produced by *B. bifidus* with the curves of the volatile acids as determined by W. H. Olmsted (in work to be published) we may conclude that the distillate probably consists largely of formic and acetic acids while that of *B. coli* appears

TABLE 4

Growth of B. coli and M. ovalis in adjusted acid broths (0.02 N) as shown by turbidity in twenty-four hours

ACID	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
Formic.................	Clear	Clear	++++	Clear	+	++++
Acetic.................	Clear	Clear	+++	Clear	+	++++
Lactic.................	Clear	++	+++	Clear	+++	++++
HCl....................	++	+++	+++	±	++	++++

TABLE 5

Viability of B. coli and M. ovalis in adjusted acid broths (0.02 N) as shown by number of days the organisms remained alive

ACID	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
Formic.................	2	7	7	2	4	4
Acetic.................	1	5	7	7*	5	5
Lactic.................	2	7	5	5	4	4
HCl....................	6	3	3	7	7	6

* Only a very few colonies were obtained on transplants after third day.

to be largely acetic. It is to be expected that little formic acid would be recovered from the *B. coli* culture as it has been shown that the formiase present breaks this substance down into carbon dioxide and hydrogen. The amount of volatile acids distilled from the culture of *M. ovalis* was so small that no opinion could be drawn as to their character, except that they are probably of the lower fatty-acid series.

Apparently, then, filtrates from these organisms contain con-

siderable quantities of organic acids and it seemed advisable to determine if media containing these acids would have any particular effect upon bacteria. Accordingly the following experiment was set up.

Normal solutions of acetic, formic, lactic and hydrochloric acids were prepared and added to 1 per cent lactose-peptone buffered-broth so that a final concentration of two-hundredth-normal was obtained for each acid. A quantity of each solution was then adjusted to pH 4.5, 5.0, and 6.0 with NaOH. An experiment similar to that with the filtrates, but using the above solutions instead of the filtrates, was then set up. The tubes containing HCl acted as controls on the organic acids. The tubes were inoculated and incubated as before. Tables 4 and 5 give the results expressed in the same manner. It may be readily seen that there is a definite inhibition of growth at pH 4.5, especially of *B. coli*, in the cultures containing formic and acetic acid, verified as before by length of life as determined by transplants. The hydrochloric-acid broth adjusted to this pH permits the bacteria to grow. Lactic acid, however, seems to have less inhibitory action than acetic and formic acids. However, as the reaction becomes more nearly neutral, the presence of the organic anion apparently makes conditions more favorable than in the tubes containing the inorganic acid. Possibly, too much emphasis should not be placed upon this phenomenon as expressed by the number of days for which the strains were viable. However, it was noted repeatedly in similar experiments. It is possible that the salts formed as these acids are neutralized have a buffering effect, but in the concentration occurring in these experiments such an effect could be but slight. The pH of these tubes was observed at the end of the experiments. At the same initial pH the final pH of the four acids varied somewhat in different experiments. With *B. coli* at pH 6.0, the organic-acid tubes in one experiment were slightly more alkaline than the HCl tube, but the difference was slight and possibly could be accounted for by errors in colorimetric reading and by the variation in abundance of growth in the tubes.

DISCUSSION

The presence of certain organic acids in culture media has much the same effect upon the growth of the intestinal bacteria studied at pH 4.5 and 5.0 as do filtrates from cultures in which these lactose-fermenting bacteria themselves have been growing. Bacterial growth is but slightly inhibited in lactose broth containing the same concentration of hydrochloric acid at these pH values. The effect of these organic acids upon the development of *B. coli* and *M. ovalis* may have some bearing upon the prevalence of these bacteria in the intestinal tract as illustrated by a comparison of their relative numbers in the feces of the artificially and breast-fed infants. The breast-fed baby has an overwhelming predominance of *B. bifidus* and in cultures prepared from fecal material under anaerobic conditions few colonies of *B. coli* and *M. ovalis* are found. If the conditions of growth in the intestine are at all similar to those in lactose-buffered broth it seems reasonable to assume that large amounts of the lower volatile acids are produced. In fact, Bosworth and his coworkers (1922) encountered formic and acetic acids in analyses of infants' stools. They endeavored to find the source of the formic acid in these stools and looked upon *B. bifidus* as a factor in its production. Their experiment, however, was based on identification of the volatile acids produced by this organism from citric acid and was disappointing in the lack of formic acid found. As has been pointed out by Kendall (1921), lactose is continually present in the intestinal tract of the normal nursling. A substitution of some other sugars invariably leads to a replacement by other organisms. No doubt, then, the formic acid found to be present in the infant's intestine is derived largely by *B. bifidus* from lactose fermentation. The fact that *B. bifidus* is practically a carbohydrate-obligate substantiates this idea. The concentration of these acids used in the experiments with the acids alone was much less than that actually produced by *B. bifidus* in the cultures. The pH of the normal breast-fed infant stool is about 5.2. It is possible that higher up in the intestinal tract the reaction is more acid. Under conditions of such acidity, according

to the above experiments, the inhibitory effect of the volatile acids would be present. Apparently *B. bifidus* in the intestine is favored by the presence of these organic acids at a high hydrogen-ion concentration and doubtless other unknown conditions, so far not reproduced in the test tube, may have a beneficial effect. Cruickshank (1925) mentions that glucose broth acidified with acetic acid inhibited *B. coli*, but allowed *B. bifidus* to develop.

On the other hand, in the artificially fed infant, one finds the predominating organism in most cases to be *M. ovalis*, with *B. coli* almost as prevalent. *B. bifidus* is usually present but to no very constant or marked degree. The pH of the feces is 6 to 8. In such cases the organic acids produced during fermentation tend to become neutralized and so fail to inhibit the intestinal forms more common in the adult intestine. It seems that organic anions tend to be inhibitory under acid conditions, but lose this power when more of the salts are present as neutrality is approached. Bial, as cited by Winslow and Lochridge (1906) noted a diminution of antiseptic action of acids when neutral salts were added. These writers considered the toxic action of organic acids due to the anion or undissociated molecule. Cohen and Clark (1919) felt that the free acetate radicle may exert a synergic effect on the disinfecting power of the hydrogen ion.

Perhaps not too much significance should be placed on the following fact; yet it is interesting to note that the filtrate from *M. ovalis* had less toxic effect than that of the other two organisms. According to the analyses of the experiments, *M. ovalis* produces the largest percentage of titratable acids, as lactic acid rather than volatile. As was mentioned before, lactic acid has less effect upon the development of bacteria than the other organic acids tested.

SUMMARY

1. *B. coli* and *M. ovalis* are inhibited in bacterial filtrates of cultures of *B. coli*, *M. ovalis* and *B. bifidus* at a pH value of 4.5 and 5.0.

2. The inhibition appears to be due to some factor in addition to that caused by an increase in H-ions.

3. Acetic and formic acids show an effect similar to that shown by filtrates, which disappears as the acids tend to become neutralized.

4. Lactic acid has less inhibitory effect than the other acids studied.

5. Under the same conditions, *B. coli*, *M. ovalis* and *B. bifidus* utilize lactose differently, giving different proportions of volatile and lactic acids.

6. The question is discussed as to the connection between the presence of large amounts of volatile acids presumably produced by *B. bifidus* in the intestine of the breast-fed infant and the inhibition of *M. ovalis* and *B. coli* in that locality. The prevalence of these last two organisms in the feces of the artificially fed infant, where the reaction is less acid, would seem to correlate with the results obtained from experiments outlined in this paper.

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THE USE OF CULTURE MEDIA MADE FROM COMMER-CIAL DRIED YEAST AS A ROUTINE SUBSTITUTE FOR MEAT INFUSION PEPTONE MEDIA

JAMES M. NEILL, JOHN Y. SUGG, LURLINE V. RICHARDSON, AND WILLIAM L. FLEMING

From the Department of Bacteriology and Immunology of Vanderbilt University Medical School, Nashville

During the past few years many batches of meat infusion pep-tone broth prepared in our laboratory have failed to grow pneumo-cocci and other kinds of bacteria that usually flourish in good batches of this medium. Because of this experience, media prepared from commercial dried yeast have been tested. Throughout a year's use they have proved to be uniformly reliable and efficient substitutes in all routine bacteriological procedures for which meat infusion peptone media are commonly used. The purpose of this paper is to report the method of preparation and the advantages and limitations of these yeast media.

METHOD OF PREPARATION

A number of different procedures have been tried, but the following methods were chosen as the simplest and most rapid.

I. Preparation of "five per cent yeast" broth

The medium found satisfactory as a routine substitute for meat-in-fusion-peptone broth was prepared by the following steps. For conven-ience, the amounts of material are expressed in terms of 10 liter lots of medium.

1. Infusion of the dried yeast. Warm 10 liters dist. H_2O to 40°C.; add 500 grams Fleischmann's dried yeast[1] to the H_2O and infuse at

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[1] The dried yeast was obtained from the Fleischmann Yeast Company. It contains no starch and consists of dried yeast cells and is the same material as that distributed commercially for feeding cattle and other stock. Four different lots

40°C. for thirty minutes; raise temperature to 50°C. and continue the infusion for ninety minutes.

2. Preliminary adjustment of pH of the infusion before boiling. Add 8 per cent (2.0 N) NaOH to the infusion until phenol red gives a faint pink when added to samples of the turbid yeast suspension (about 40 cc. 2.0 N NaOH are usually required). Add 30 grams $Na_2HPO_4 \cdot 12$ H_2O previously dissolved in 100 to 200 cc. of warm H_2O.

3. Boiling and preliminary filtration of yeast infusion. Raise temperature to boiling; stirring is necessary when the temperature approaches boiling point. Boil for one to three minutes. Filter through paper.

4. Adjustment of volume. Add H_2O to compensate for loss due to evaporation and filtration, until volume is 9.5 liters.

5. Final adjustment of pH. The broth is perfectly clear after the filtration in step 3, and colorimetric tests always show the pH to have dropped below 7.0. Adjust by addition of NaOH until pH is from 7.2 to 7.4. Add 150 grams $Na_2HPO_4 \cdot 12$ H_2O previously dissolved in 500 cc. warm H_2O. The addition of this phosphate usually raises the pH to about 8.0 with media prepared from most lots of the dried yeast. If a final pH of 7.8 is desired, the broth should be readjusted by addition of alkali, or acid if it is not pH 8.0 after addition of the phosphate at this stage. (We prefer to adjust the pH with NaOH added in the order described to a point sufficient to enable the last addition of phosphate to raise the pH to 8.0. This point (usually pH 7.2 to 7.4) varies to some extent with different lots of yeast, but is fairly constant in different batches prepared from the same lot of dried yeast.)

6. Precipitation at 120°C. before final filtration. Distribute the adjusted broth into large flasks and autoclave five minutes at 120°C.

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have yielded uniformly good culture media, so that it is probable that all similar material will give good results. There was some variation in the buffer curves and in the content of fermentable substance in media prepared from different lots of the dried yeast, but none of these variations introduced any significant complications. Apparently, the yeast should be obtained as a reasonably fresh preparation; although lots of dried yeast that had been in the laboratory for over six months proved satisfactory, one lot that had been stored for about three years in a warm room furnished inferior media.

The dried yeast from which most of the media has been prepared, was in the form of coarse granules. Broth made from one lot of finely powdered yeast seemed equally good from the standpoint of its nutrient qualities, but the media made from the powdered material had the disadvantage of slower filtration than that made from the granular material.

This causes precipitation of material that would otherwise be precipitated during the final sterilization.   The precipitate is well separated from the clear supernatant and the medium filters as rapidly as ordinary broth. This heating usually causes a drop in pH from 8.0 to 7.9.

7. Final sterilization. Distribute the filtered broth in the desired containers and sterilize twelve to fifteen minutes at 120°C.   This usually causes the pH to drop to 7.8.

II. Preparation of agar medium with "five per cent yeast" broth as base

1. Preliminary softening of agar.   Add 250 grams agar to about 2 liters H_2O and let it soak until time for its addition to the medium.

2. Preparation of yeast broth base.   Proceed as in steps 1, 2, and 3 described under I.   (Use 10 liters H_2O as the decrease in volume will compensate for the H_2O added to the agar.)

3. Adjust volume of broth, if decrease in volume after filtration has reduced it lower than 7.5 liters.

4. Adjustment of pH.   Raise pH to about 7.4 to 7.5.   Add 150 grams phosphate dissolved in about 500 cc. H_2O as in step 5 in I.   This should bring reaction to at least pH 8.1, and if final pH of 7.8 is desired, the reaction should be readjusted if it is now below pH 8.0.

5. Addition of agar.   Add agar softened by previous soaking in step 1.

6. Solution of agar and precipitation before final sterilization.   Autoclave for fifteen minutes at 120°C. instead of the 5 minutes employed in step 6 of I, in order to dissolve the agar.   Filter through cotton and test pH which should now be pH 7.8 to 7.9.

7. Final sterilization.   As in analogous step in I.

III. Preparation of "twelve per cent yeast" broth

This medium is prepared by essentially the same procedure as described in I for "5 per cent yeast" broth with the exception that the amount of dried yeast is increased to 120 grams per liter.   The additional amount of yeast makes the preparation of this medium much more time-consuming, for filtration which is rapid with the "5 per cent" medium is slow with the "12 per cent" medium.

IV. Preparation of agar medium with "twelve per cent yeast" broth as base

Agar medium may be prepared by the addition of 2.5 per cent agar to the "12 per cent yeast" broth.

The uniform reliability of every batch of "5 per cent yeast" broth is sufficient justification for its substitution for meat infu-

sion peptone media for routine use in laboratories which have difficulty in the preparation of uniformly satisfactory media from meat. However, in addition to the advantage of reliability, the yeast broth possesses the advantage of cheapness. The total cost of the materials for one liter is 9 cents (7.6 cents for the yeast and 1.4 cents for the phosphate), or approximately the same as that of the 10 grams of peptone contained in one liter of meat infusion peptone media. Hence, by the use of yeast broth in place of meat infusion peptone broth, one saves the entire cost of the meat which varies from 15 to 30 cents per liter. The "12 per cent" media are described because for some special purposes they possess the advantage of a somewhat more rapid and luxuriant bacterial growth. In many instances, the richer medium is a disadvantage and in general practice, the advantage of the "12 per cent" over the "5 per cent" medium is not sufficient to justify the greater labor and expense involved in its preparation.

FERMENTABLE CARBOHYDRATE, BUFFER VALUE, AND FINAL PH OF CULTURES GROWN IN THE DESCRIBED YEAST MEDIA

The amount of reducing sugar in "5 per cent yeast" broth is insignificant as it is not detectable in tests of 0.5 cc. of the broth layered on 2.0 cc. of Benedict's solution; slight traces are present in some batches of the "12 per cent" broth. The amount of reducing sugar, is never sufficient to account for the growth-promoting properties of the yeast broth. Tests for glycogen proved positive in all lots of yeast broth. The presence of fermentable carbohydrate has been recognized by other workers in the preparation of different yeast media and some of them have employed preliminary autolysis of the yeast as a means of removing the fermentable substance. Our main purpose, however, was to obtain a medium that was simple in preparation and preliminary autolysis of the yeast seemed to complicate the method to too great an extent.

The amount of phosphate in the medium is sufficient to compensate for the production of a considerable amount of acid, and in the "5 per cent" broth none of the common bacteria reach a

final pH sufficiently acid to interfere with most routine bacterio-
logical procedures. (The production of acid varies to some extent
with the lot of dried yeast from which the medium is prepared,
but the following pH values represent that obtained in most of
the batches of "5 per cent yeast" broth that we have made:
Pneumococci, pH 6.6 to 7.0; scarlet fever and other hemolytic
streptococci, 6.3 to 6.7; staphylococci, 6.7 to 7.0; diphtheria
bacilli, 7.2 to 7.6; anthrax bacilli, 6.6 to 6.9; Friedländer, colon
bacilli, and paratyphoid, 6.3 to 6.7; typhoid, 6.6 to 6.9; Flexner
dysentery, 6.3 to 6.4; Shiga dysentery 6.7 to 6.8; tetanus, 6.8 to
7.2; Welch bacillus, 6.5 to 6.8; vibrion septique, 6.3 to 6.6.)
Hence one can simply accept the production of some acid as a
property of the medium that does not interfere with its routine
use. Rather than to complicate the method of preparation by
attempts to remove the source of acid, it seemed preferable to
use another culture medium for any purpose in which the acid
production was a real disadvantage. The amount of acid pro-
duced in the "12 per cent yeast" broth, however, is significant;
with some bacteria, the pH drops to below 6.0; pneumococci,
for example, usually giving a final pH from 5.6 to 6.0. For some
purposes, however, even with pneumococci, this disadvantage
can be circumvented by using the cultures at an early growth
stage before the final high acidity is reached.

TESTS OF POSSIBLE SEROLOGICAL COMPLICATIONS

Since Fleischmann's dried yeast was the source of Mueller's
preparation of the specific yeast carbohydrate (Mueller and
Tomcski, 1924), some of the same serologically reactive substance
must be contained in the described yeast broth. This suggested
two possible sources of danger. First, that immunization with
yeast broth cultures of bacteria might give rise to anti-yeast
precipitins reactive with the broth itself. Second, that the
reactive yeast carbohydrate while derived from yeast, might be
chemically related to the specific *S* substance of certain bacteria
and consequently cause some serological reaction between yeast
broth and the antibacterial serum.

We have investigated both these possibilities. The first appears to be unimportant as a source of error. Six rabbits have been immunized with the described yeast broth; two of them with 4 courses including 24 injections of 10 cc. of the broth. None of the animals developed any detectable traces of precipitins against the yeast broth. Apparently, the dissociated *S* substance which Mueller found to be devoid of antibody-invoking properties represents the only yeast antigen (haptene) present in significant amounts in the broth. If use of the yeast broth is limited to routine purposes, the possibility of the presence of traces of other yeast antigens is not important.

The second possibility would be encountered in the agglutination of cultures grown in yeast broth against an antibacterial serum that might happen to include precipitins reactive with the *S* substance contained in yeast broth. That this is a real danger is due to two facts: (1) that Fleischmann's yeast possesses a certain serological relationship to Type II pneumococci; (2) that the serum from many normal rabbits possesses agglutinins for cultures of the *yeast cells* grown in any kind of broth. (These inter-reactions have been reported by Sugg and Neill (1929)). However, while some normal rabbit sera and Type II antipneumococcus horse serum do agglutinate *yeast cells*, none that we have tested precipitate the described "5 per cent yeast" broth. The yeast broth is precipitated by antiserum prepared by immunization with yeast cells (as would be expected from Mueller's work with yeast and from Avery and Heidelberger's work with pneumococci), but not by any of over 100 antibacterial antisera that we have tested. Hence, unless one attempted to agglutinate bacterial cultures grown in yeast broth against an anti-yeast-cell serum, the use of yeast broth cultures of bacteria is not likely to give false-positive agglutination results. We have also tested yeast broth cultures of many different kinds of bacteria against Type II antipneumococcus serum and normal and immune rabbit sera that do agglutinate yeast cells, and none of them have ever agglutinated the heterologous bacteria. Thus, control tests of the sterile broth itself against the agglutinating serum should eliminate any source of error in the agglutination of yeast broth cultures of bacteria.

The yeast broth grows pneumococci so well that it seemed particularly adapted for use in routine diagnostic typing. However, the fact that the yeast possesses some serological relationship to Type II pneumococci made it necessary to make many control experiments to determine whether or not false agglutination would occur with heterologous types of pneumococci grown in the yeast broth. In none of these experiments

was any heterologous type (Type I, III, IV) grown in yeast broth agglutinated or precipitated by Type II antiserum.   From the result of these tests, pneumococcus cultures grown in yeast broth seem permissible for use in routine diagnostic typing, provided control tests are made to prove that the Type II serum does not precipitate the yeast broth medium itself.

USES OF THE YEAST MEDIA

The described yeast media have been used for some time in the clinical laboratory of bacteriology of the Hospital and have been found suitable for all routine procedures in which meat infusion peptone media are commonly used.   The combined use of flasks containing 200 to 300 cc. of the "5 per cent yeast" broth and of blood plates from yeast agar have sufficed for the culture of most infectious material.   The same "5 per cent yeast" broth and agar in deep tubes sealed with vaseline have been equally satisfactory in the culture of anaerobes from clinical material. Blood agar plates prepared from the "5 per cent yeast" medium have given uniform satisfaction, not only from the standpoint of nutrient properties but also from the standpoint of the properties (hemolysis and methemoglobin production) utilized in the differentiation of the Gram-positive cocci.

Although peptone is not added to yeast broth, it serves for the production of lysins (*B. Welchii*, tetanus bacillus and streptococcus) and toxins (diphtheria and tetanus).   While not particularly interested in substituting the yeast broth for the usual meat infusion peptone medium in the production of toxin, we have regularly obtained more potent diphtheria toxin with the routine "5 per cent yeast" broth with all strains tested than under similar conditions with the best meat infusion peptone broth that could be prepared in this laboratory.

The "5 per cent yeast" broth has proved useful as a medium for pneumococcus cultures in typing.   As a means of obtaining more prompt results in diagnostic typing, the "12 per cent yeast" broth has the advantage of more rapid growth.   This medium, if inoculated with the heart's blood of a mouse, usually gives a culture of sufficient growth for typing and biling within 3 to 5

hours after inoculation. The "12 per cent yeast" broth, how-
ever, has the disadvantage of acid production below pH 6.0 if
cultures are allowed to reach their maximum growth. In
emergencies (which are the only times in which the "5 per cent"
broth does not give sufficiently rapid growth) the cultures in
"12 per cent" broth can be kept under constant observation and
be tested before they become acid. (The necessity of controls
against the possibility of serological reaction between the yeast
broth and Type II antipneumococcus serum has been discussed
under "Tests of Possible Serological Complications.")

<div align="center">COMMENT</div>

A description has been given of the method of preparation of
culture media from commercial dried yeast. Media prepared
from yeast have been reported by other workers; in most cases,
the medium has contained peptone or other substances in addition
to the yeast; in other cases, the method of preparation has been
somewhat too involved for a routine medium. Our object was to
obtain a reliable culture medium, each batch of which could be
depended upon for the growth of all kinds of bacteria that will
grow in good batches, but not in poor batches, of meat infusion
peptone media. At the same time, it was desired to keep the
method simple enough to permit the preparation of the media in
a reasonably short time by the ordinary technician. The
described yeast media meet both these requirements and after a
thorough test, we believe that they possess two definite advan-
tages over meat infusion peptone media for all routine bacterio-
logical procedures. The first advantage is reliability, in that each
batch will support growth of pneumococci and other bacteria that
fail to grow well in any batch of meat extract peptone broth or in
a poor batch of meat infusion peptone media. Not a single one of
50 batches made from 4 different lots of the dried yeast has
proved unsatisfactory. The second advantage is economy; the
cost of the yeast and the phosphate is no greater than that of the
peptone used in making meat infusion peptone media, and hence,
the entire cost of the meat is saved. That the yeast media are
as easy, or easier to prepare than meat infusion media, and that

they can be sterilized by a single process in the autoclave are factors that fit them for routine use.

Although the described yeast media have these definite advantages it is necessary to accept certain limitations to their use. The first is the production of acid by some bacteria. This complicates the use of yeast broth as a base for the special carbohydrate media employed in testing the fermenting properties of bacteria. However, in the usual laboratory, carbohydrate fermentation tests are used principally in the study of the colon-typhoid group of bacteria, which grow well in meat extract-peptone media. Consequently, we have made no attempt to use the yeast broth for this purpose, since we have considered it as a substitute for meat infusion peptone media and not for the cheap and easily prepared meat extract peptone broth. The second limitation is the possibility of complications arising from the fact that the yeast broth contains detectable amounts of the serologically reactive yeast carbohydrate described by Mueller and Tomcski (1924). However, the experiments carried out in this connection showed that errors are not likely to result from the routine use of yeast broth cultures in properly controlled agglutinination and precipitin tests. Nevertheless, it is probably inadvisable to use yeast broth cultures in immunological investigations (particularly in problems connected with pneumococci) until more is known of the serological relationships of yeast to bacteria.

SUMMARY

This paper describes the method of preparation of culture media from commercial dried yeast, and reviews their advantages and limitations. The "5 per cent yeast" broth and agar have been found satisfactory for all routine purposes for which meat infusion peptone media are commonly employed. The yeast media are easy to prepare and possess the definite advantages of uniform reliability and of low cost (9 cents per liter of broth). Their use is especially recommended for the routine culture of infectious material in clinical bacteriological laboratories.

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RELATIONSHIPS OF THE ENCAPSULATED BACILLI WITH SPECIAL REFERENCE TO BACT. AEROGENES[1]

PHILIP R. EDWARDS

From the Department of Veterinary Science, Kentucky Agricultural Experiment Station, Lexington, Kentucky

The purpose of this paper is to present the results of work on the encapsulated bacilli and to discuss the relationships between some of the members of this group. This work is the outgrowth of a study of encapsulated bacilli found in cases of genital diseases in horses. These organisms have been described by Dimock and Snyder (1925), Dimock and Edwards (1927) and Edwards (1928). While these organisms were being compared with encapsulated bacilli isolated from human sources it was noted that certain cultures of *Bact. aerogenes* were closely related to the cultures from equine sources and to some of the cultures of human origin. This relationship seemed worthy of further study; so a number of cultures of *Bact. aerogenes* were collected and compared with the human and equine cultures in our possession.

SOURCE OF CULTURES

The cultures of Friedländer and granuloma bacilli which have been used in this work were obtained from several investigators who forwarded them to us. With one exception the strains of *Bact. aerogenes* were obtained in the same way. One of the cultures of *Bact. aerogenes* and the organisms from genital diseases in horses were isolated in this laboratory. Following are given the designations of the cultures and the sources from which they were obtained.

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[1] Published with the approval of the director of the Kentucky Agricultural Experiment Station.

E1, E2, E8—isolated from uteri of infected mares F3, F5, F6, F8, F9, F10, F11, F13, F18, F20, F22, F23, F24, F25, F28, FR— Pneumonia, human

F1, F2—extirpated appendix, human

F21—extirpated tonsil, human

F26—chronic colitis, human

F27—chronic cystitis, human

F15—mitral regurgitation, human

F19—pleuritis, human

G2, G6—inguinal granuloma, human

AB—*Bact. aerogenes*, milk

AR, A9, A10, A14, AH9—*Bact. aerogenes*, feces

A3, A5, A7, A8, A11, A12, A50, A65, A70, A73, A97, A111, A112, A113, A114, A115, A153, AM8C—*Bact. aerogenes*, soil

A6, A13—*Bact. aerogenes*, water

The cultures E1, E2 and E8 represent a group of sixty-five encapsulated organisms recovered from cases of metritis in mares. Forty of these sixty-five strains have been studied and found identical in morphological, cultural, biochemical and serological properties. All are identical with type B of the Friedländer bacillus as described by Julianelle (1926). Since these cultures exhibit the same properties only three of them are included in this study.

CULTURAL CHARACTERISTICS

The cultural characteristics of the majority of the organisms included in this study are those generally attributed to the members of the *B. mucosus* group. An abundant, dirty white to slightly yellow growth which is moist, glistening and slimy occurs on agar slants. Agar colonies are large, raised, round and entire. The bacteria are Gram negative, non-sporing, non-motile rods which do not liquefy gelatin. Most of the organisms produce wide capsules on artificial media. Some of the cultures which have been grown on artificial media for long periods no longer produce the capsules typical of this group. Such strains do not exhibit a mucoid growth on agar and their virulence is reduced. This lack of virulence, loss of capsules and failure to exhibit a mucoid growth on agar occurred among all the groups studied,

the Friedländer, equine and granuloma strains as well as the cultures of *Bact. aerogenes* being affected in this way.

METHODS

The methods used in fermentation tests, immunization of rabbits, agglutination, agglutination absorption, and precipitin tests are those employed by Edwards (1928). The protein antigens used in the precipitin tests were prepared by the method of Julianelle (1926b).

TABLE 1

Biochemical reactions

	AMMONIA PRODUCTION	NITRATE PRODUCTION	INDOL PRODUCTION	METHYL RED	VOGES-PROSKAUER	GROWTH IN URIC ACID	GROWTH IN CITRATE	ACTION IN LITMUS MILK
E1, E2, E8, F6, F26, A3, F11, A5, A7, A10, A13, A50, A65, A113, A115, A150, A153 AB, AH9, G6.	+	+	−	−	+	+	+	AC
F3, F5, F9, F15, F18, F19, F23, F25, F28, A6, A11, A14, A114..............	+	+	−	−	+	+	+	A
F2, F8, F10, F24, F27, FSc.	+	+	−	+	−	+	+	A
F21, A97, A112............	+	+	−	+	−	+	+	AC
F1.....................	+	+	−	+	−	−	+	A
F13, FR..................	+	+	−	+	−	−	−	A
F20.....................	+	+	−	−	+	−	−	A
F22.....................	+	+	−	−	+	−	−	Al
AR, A8, A9, A111.........	+	+	+	−	+	+	+	AC
A73, AM8C...............	+	+	+	−	+	+	+	A
A70.....................	+	+	+	+	−	+	+	A
A12.....................	+	+	−	+	−	+	−	A
G2	+	+	−	−	−	+	+	AC

BIOCHEMICAL REACTIONS

The cultures studied have been subjected to the tests usually applied to the organisms of the colon-aerogenes group. The strains have been tested for indol production, growth in citrate and uric acid media, nitrate reduction, production of ammonia, action in litmus milk and response to the methyl red and Voges-Proskauer tests.

TABLE 2

Fermentative reactions

	GLUCOSE	LEVULOSE	GALACTOSE	LACTOSE	SUCROSE	GLYCEROL	DULCITOL	MANNITOL	SORBITOL	ARABINOSE	XYLOSE	RAFFINOSE	MALTOSE	ADONITOL	SALICIN	INULIN	DEXTRIN	SOLUBLE STARCH
E1, E2, E8, F3, F5, F8, F6, F13, F15, F19, F20, F21, F24, F25, F26, F28, AB, A3, A13, A14, A50, A112, A113, A114, G2	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
F2	A	AG	AG	AG	O	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
F23	A	AG	AG	O	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
F10, F27, A150, AH9	A	A	A	A	A	A	AG	AG	AG	A	A	A	AG	A	AG	O	AG	AG
Sc	O	A	A	A	O	O	A	A	AG	A	A	A	A	A	A	O	A	A
F1	A	A	A	A	A	A	O	A	A	A	A	A	A	A	O	O	O	O
F11	AG	O	O	O	O	O	O	A	O	A	A	O	O	O	A	O	A	A
F18	AG	AG	A	A	A	A	O	A	A	A	A	A	A	A	O	O	O	O
F22	AG	AG	AG	AG	AG	AG	O	AG	O	AG	O	O	O	O	A	O	AG	AG
G6	AG	AG	AG	AG	AG	AG	O	AG	A	AG	A	A	A	AG	AG	AG	AG	AG
AR, A6, A9, A97	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
A5, A7	AG	AG	AG	A	AG	O	AG	AG	AG	AG	AG	AG	AG	O	AG	O	AG	AG
A10	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
A11, A150, AH9	AG	AG	AG	AG	AG	O	O	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	AG
A12	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	O	AG	O	AG	O
A65	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
A115	AG	AG	AG	AG	AG	O	O	AG	AG	AG	AG	AG	AG	O	AG	O	AG	AG
A153	AG	AG	AG	AG	AG	O	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	O
A8, A70, A73, A111	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG

From table 1 it can be seen that the organisms studied are divisible into several groups on the basis of their reactions to the tests employed. These groups do not appear to be significant however, since members of the various species are dispersed through several groups. It is evident that this combination of tests cannot be employed in the separation of the various species. If the results of each of the tests be considered separately it may be seen that not one of the tests employed can be used to differentiate the various species.

FERMENTATIVE REACTIONS

The cultures studied were tested for their ability to form acid and gas from a number of fermentable substances. An examination of table 2 shows that the cultures of the various types cannot be separated by their action upon the fermentable substances used. Certain groups are established by the fermentation tests but the various species are dispersed throughout these groups. The groups established by the fermentation reactions do not agree with those established by the biochemical reactions given in table 1, nor, as will be shown later, are they related to the groups established by the serological tests. Both the cultures of *Bact. aerogenes* and the Friedländer bacilli are rather variable in their fermentative characters.

SEROLOGICAL REACTIONS

The early literature regarding the serological properties of the Friedländer group is very confusing. Later workers have successfully classified the organisms by serological methods. Toenniessen (1914) suggested that the capsular substance was antigenic in character and that Friedländer bacilli were capable of exhibiting two antigenic complexes, one residing in the capsule and the other in the endoplasm of the bacterium. Toenniessen (1921) later demonstrated that the capsule of these organisms was a galactan. Heidelberger, Goebel and Avery (1925) derived a nitrogen-free polysaccharide from Friedländer bacilli which was capable of giving positive precipitin tests with homologous immune serum. Julianelle (1926, 1926a, 1926b) by applying

these facts to the classification of Friedländer strains has established four serological groups among the encapsulated bacilli. These he has designated as types A, B, and C, and Group X. Types A, B and C are specific while group X is composed of a number of heterologous strains.

Julianelle demonstrated that organisms which produced capsules were agglutinated by serum derived from an organism of the homologous type but were not affected by sera derived from organisms of heterologous types. The manner in which these organisms were clumped was characteristic, agglutination resulting in the formation of a compact, voluminous disc at the bottom of the tube. Organisms which no longer produced capsules, or bacilli which had been stripped of their capsule by chemical methods, did not exhibit this type specificity. They were agglutinated by the immune sera of both the homologous and heterologous types. The character of the agglutination was changed. Instead of the formation of a compact disc, agglutination resulted in a fine, powdery precipitate.

In the present investigation the Friedländer, granuloma, and equine organisms have been compared with the cultures of *Bact. aerogenes* using agglutination, precipitin, and agglutinin absorption tests in an effort to determine their relationships.

AGGLUTINATION

Cultures E1, F6, FSc, G2, G6, and A3 were used to prepare antisera. Using these sera, agglutination tests were set up in dilutions ranging from 1 to 20 to 1 to 2000. Two main types are established by the agglutination tests. The first group composed of strains E1, E2, E8, F6, F26, FR, AB, A3, A13, A113, and A114 is identical with type B of the Friedländer bacillus as described by Julianelle (1926). These organisms have the cultural characters generally attributed to the soil types of *Bact. aerogenes*. It will be noted that in this type are included five strains of *Bact. aerogenes*, three isolated from soil, one from water and one from milk.

The second large group is composed of strains F3, F5, F8, F9, F18, F19, F20, F21, F23, F24, F25, F28, FSc. These strains are identical with type A of the Julianelle.

A third group established by the agglutination test is composed of three cultures, G6, A70, and A73. G6 is a culture received from Dr. J. C. Small. It was recovered from a local lesion of inguinal granuloma. The cultures A70 and A73 are soil strains of *Bact. aerogenes* received from Dr. S. A. Koser.

In addition to the specific agglutination observed in these tests several instances of group or non-specific agglutination occurred among the aerogenes strains. This was most evident in the cases of strains A12 and A14. Julianelle (1926a) attributed group agglutination among the Friedländer bacilli to the absence of capsule formation. The same cause is probably operative in the case of *Bact. aerogenes*. The cultures which were acted upon by the group agglutinin exhibited a rather dry growth on agar and no capsules could be demonstrated in these cultures.

During the course of this work the action of hydrolysis upon agglutination of the encapsulated bacilli has been studied. This method, first used by Porges (1905), and since employed with more or less success by a number of investigators, consists of treating suspensions of encapsulated bacilli with one fourth volume of 0.25 N HCl, heating at 80°C. for fifteen to thirty minutes and neutralizing the suspension by the addition of NaOH. This treatment is designed to strip the organism of its capsule and render it easily agglutinable. Beham (1911) stated that this method often rendered the bacilli spontaneously agglutinable. The results of our work with hydrolyzed suspensions have, on the whole, been unsatisfactory. Many organisms thus treated became spontaneously agglutinable. Still other strains, after they had been so treated, were not affected by sera known to be potent in group agglutinins.

However, by varying the temperature and the time of exposure to acid we have been able to render a number of the strains susceptible to group agglutinin. We have not been able to distinguish *Bact. aerogenes* from the other types by this method. We have found that, after hydrolysis, *Bact. aerogenes* strains are agglutinated by Friedländer antisera to about the same extent as the equine organisms and Friedländer and granuloma bacilli.

The tests performed with type specific precipitin antigen confirm the results of the agglutination tests in every case. The cultures which were agglutinated by sera derived from strains E1 and F6 gave strongly positive precipitin tests with these sera while no precipitation resulted with sera derived from strains G6 and Sc. Also, strains A70 and A73 gave strongly positive tests with serum derived from strain G6 but caused no precipitation when placed in contact with the sera of other types. The strains composing type A of Julianelle were also specific in their precipitin reactions.

In the precipitin tests using protein antigens the proteins derived from strains of *Bact. aerogenes* yielded strongly positive tests with antisera derived from cultures of Friedländer and granuloma bacilli. It was not possible to distinguish between the organisms of the various types in this way. Julianelle (1926b) has observed the precipitation occurring when Friedländer antisera are placed in contact with protein derived from *Bact. aerogenes*. This reaction indicates the close serological relationship of the proteins of these bacilli.

The results of the agglutinin absorption tests further substantiate the relationships established by the agglutination tests and precipitin tests. In every case in which type specific agglutination occurred the strain so agglutinated was able to exhaust the type serum of agglutinins completely. Thus, strains E2, E8, F6, F26, FR, AB, A3, A13, A113, and A114 effected a complete removal of agglutinins active on strain E1 from antiserum derived from that strain. They were also able completely to remove the agglutinins active on strain F6 from antiserum derived from F6. Further, these strains caused a complete removal of agglutinins from A3 antiserum. This group of organisms was inactive when used to absorb the sera of other types.

Similarly strains A70 and A73 completely exhausted antiserum G6 of agglutinins for the serum strain. They were without

effect when used to absorb antisera derived from strains E1 and FSc.

The group of Friedländer strains composing type A were also apparently of homogeneous antigenic structure since they all exhausted serum FSc of agglutinins active upon the homologous strain.

The strains which were agglutinated non-specifically were unable to completely remove agglutinins from any of the antisera.

PATHOGENICITY

Baerthlein (1918) and Toenniessen (1921) have demonstrated that the virulence of Friedländer bacilli for the laboratory animals is dependent upon capsule formation. In a previous publication, Edwards (1928), we have found that the ability of organisms of this group to cause metritis in mares is also dependent upon abundant capsule formation.

In studying the effect of cultures of *Bact. aerogenes* on laboratory animals we have found that those cultures which produced wide capsules and a mucoid growth on artificial media were uniformly virulent for the laboratory animals. Using the technique described by Dimock and Edwards (1926), it has been found possible to produce metritis in mares through the introduction of *Bact. aerogenes* into the uterus. However, only those strains which were vigorous capsule producers were able to become established.

No constant differences in pathogenicity of the various types have been observed.

DISCUSSION

It is evident from the results obtained in this study and from the results of other workers that such tests as ammonia production, nitrate reduction, indol formation, growth in uric acid and citrate media, and the methy red and Voges-Proskauer tests are of no value in the differentiation of the various types of encapsulated bacilli.

The action of the organisms when grown in milk has been employed by many writers to set apart *Bact. aerogenes* from the

other members of the group. Basing their statements on the work of Wilde (1896) and Claremont (1902) they characterize *Bact. aerogenes* as a milk coagulator, while Friedländer's bacillus is described as causing no coagulation in milk. However, the results of other workers have been contradictory. Fricke (quoted by Abel and Hallwacks (1913) found a striking variation in the ability of strains isolated from pathological sources to coagulate milk. Fitzgerald (1914) observed that certain strains of Friedländer's bacillus were able to coagulate milk while other strains caused no coagulation. Even when the utmost care was used in the tests, these differences were apparent and Fitzgerald warns against the use of coagulation of milk as a differential test. Small and Julianelle (1923) studied 13 cultures of Friedländer bacilli and found that 5 of the strains coagulated milk.

In the present study we have found that four strains of Friedländer bacilli cause a coagulation in milk. It is noteworthy that two of the Friedländer strains used in this study which did not produce coagulation in milk had been previously studied by Small and Julianelle and found to be milk coagulators. Among the aerogenes cultures studied there were seven which caused a permanent acidity in milk but produced no coagulation. Others coagulated the milk only after four to ten days incubation. In addition it must be remembered that 40 cultures of equine origin, which are identical with type B of the Friedländer bacillus, rapidly coagulate milk. In view of these facts it would seem that coagulation of milk must be accepted with reservations, if it is accepted at all, as a means of differentiating *Bact. aerogenes* from the other members of the encapsulated group.

The fermentative characters of the encapsulated bacilli have been studied by a number of workers and several classifications have been based upon the action of these organisms on fermentable substances. These studies, however, are far from being in agreement. The greatest controversy has centered about the ability of Friedländer's bacillus to ferment lactose. Many writers contend that this organism produces acid and gas from glucose and sucrose but does not attack lactose. Friedländer (1882, 1883, 1884) did not mention the action of the organisms

which he isolated on milk or lactose. Strong (1889) working with strains which were presumably lineal descendants of Friedländer's original cultures found that the organism produced no acid or gas from lactose. Grimbert (1895) stated that Friedländer's bacillus was able to produce acid from lactose. Wilde (1896) reported that the strains of Friedländer bacilli which he studied formed acid from lactose. Nicolle and Hebert (1897) found that Friedländer's bacillus formed acid and gas from lactose. Russ (quoted by Abel and Hallwachs (1913) stated that Friedländer's bacillus fermented lactose. Lehman and Neumann (1901) reported that Friedländer's bacillus fermented lactose with the production of acid and gas. Claremont (1902) found that all of the ten Friedländer strains which he studied produced acid from lactose. Nine of these strains formed perceptible gas. Perkins (1904) classified the encapsulated bacilli according to their action on glucose, lactose, and sucrose. Those strains which were able to ferment glucose and sucrose but did not attack lactose he designated as Friedländer's bacillus. A culture from Kral, supposedly a descendant of the original strain of Friedländer, did not attack lactose. MacConkey (1905) on the contrary, found that a transplant obtained indirectly from the Kral culture produced acid in lactose broth. Fitzgerald (1914) found that six of seven Friedländer strains examined attacked lactose and offered evidence that fermentation could not be relied upon in the classification of the encapsulated bacilli. Coulter (1917) studied eleven strains of Friedländer bacilli and found that none of them fermented lactose and that they formed a uniform serological group. Castellani and Chalmers (1919) in their classification of the colon-typhoid group inserted the genus Encapsulatus. The type species, *Encapsulatus pneumoniae*, Friedländer 1883, was described as forming acid and gas from glucose and sucrose and acid alone from lactose. *Encapsulatus aerogenes* was said to ferment glucose, lactose, and sucrose with the formation of acid and gas. Bergey (1921) stated that Friedländer's bacillus formed acid and gas from lactose. Small and Julianelle (1923) found that eleven of thirteen Friedländer strains which they studied produced acid from lactose. In addition five of the cultures which

they studied produced gas from this sugar.   Perkins (1925) supplementing his former work, again classified the Friedländer bacillus as a non-lactose fermenter.

In this work we have studied twenty-two strains of Friedländer bacilli.   With the exception of three strains, all of these organisms formed acid from lactose and, in addition, sixteen of the twenty-two cultures formed a perceptible amount of gas.

The conflicting results obtained by various workers, each believing himself to be working with the original strain of Friedländer's bacillus, are worthy of note.   It seems from a review of the literature that most of the cultures of excapsulated bacilli isolated from cases of pneumonia and from the respiratory tract are able to ferment lactose.   If lactose fermentation is used as a criterion for the differentiation of species it will result in the separation of organisms serologically identical into different species.   Such a procedure hardly seems justified when the majority of the organisms labelled as Friedländer's bacillus are lactose fermenters.

The agglutination of aerogenes strains by Friedländer sera was noted by Claremont (1902).   However, the same strains of *Bact. aerogenes* when used to prepare serum did not agglutinate any of his strains of Friedländer bacilli.   It is highly probable that this agglutination was non-specific or group agglutination. Bertarelli (1906) also has observed the agglutination of aerogenes by Friedländer sera.   Julianelle (1926a) calls attention to the close relationships of aerogenes and Friedländer proteins.   He explains the non-specific agglutination of capsule free strains of *Bact. aerogenes* on the basis of this relationship between the proteins of the two organisms.

It is the opinion of the writer that specific agglutination occurring between Friedländer's bacillus and *Bact. aerogenes* has not been heretofore recorded.   Tomcsik (1927) has isolated a protein free carbohydrate-like specific substance from strains of *Bact. aerogenes*.   This substance, however, did not prove reactive with anti-Friedländer sera.

In the present work it has been found that three strains of *Bact. aerogenes* isolated from soil, one strain isolated from water

and one strain isolated from milk are identical with type B of the Friedländer bacillus as described by Julianelle (1926). Two other strains of *Bact. aerogenes* have been found to be antigentically identical with a strain of the granuloma organism. Sera for only three types of the encapsulated bacilli were used in this study. If sera were prepared for the other types of Friedländer bacillus and the strains of *Bact. aerogenes* tested with these, it is probable that still further relationships would be established. Using only the three type sera, it has been found that seven of 26 cultures of *Bact. aerogenes* are identical in their antigenic structure with certain of the Friedländer and granuloma bacilli.

It will be noted that no aerogenes cultures were found to be identical with type A of the Friedländer bacillus. This type, apparently, is most commonly found in human infections, while type B seems to be found most frequently in the lower animals.

Bergey (1921) has classified *Bact. aerogenes* under the genus Aerobacter while Friedländer's bacillus is classified under the genus Encapsulatus. Perkins (1925) includes both of these organisms in the same genus, Encapsulata, and differentiates them on the basis of lactose fermentation. Weldin (1927) places *Bact. aerogenes* in the genus Aerobacter and places Friedländer's bacillus in the genus Proteus. Castellani and Chalmers (1919) place the two organisms in the same genus, Encapsulatus, and differentiate them by the formation of gas from lactose.

From the results obtained in this study it seems that *Bact. aerogenes* is so closely related to Friedländer's bacillus and the bacilli isolated from lesions of inguinal granuloma that it should certainly be placed in the same genus as the remainder of the encapsulated bacilli. There are no constant differences between the strains which we have received labelled as Friedländer's bacilli and *Bact. aerogenes*. Furthermore we have found no distinguishing characters which might be used to separate these organisms into two or more species.

CONCLUSIONS

1. The organisms which we have received from various sources labelled Friedländer bacilli cannot be distinguished from *Bact.*

aerogenes and the other members of the encapsulated group by action on milk or fermentative characters.

2. Five cultures of *Bact. aerogenes* isolated from soil, water and milk have been found to be culturally, biochemically, and serologically identical with type B of the Friedländer bacillus as described by Julianelle. Two cultures of *Bact. aerogenes* have been found serologically identical with a strain of the granuloma bacillus.

3. *Bact. aerogenes* is so closely related to the other encapsulated forms that they should be classified in the same genus. No constant differences have been observed which could be used to separate the organisms into two or more species.

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PRELIMINARY REPORT ON A METHOD FOR THE DIFERENTIATION OF L. ACIDOPHILUS FROM L. BULGARICUS

WALTER L. KULP

Department of General Bacteriology, Yale University

At the present time there appears to be much confusion in the classification and identification of the lactobacilli, particularly with reference to *L. acidophilus* and *L. bulgaricus*. During recent years these two species have received a considerable amount of study from research workers in this field, and several tests have been advocated as aids in the identification of typical strains of these bacteria.

Morphologically and culturally. *L. acidophilus* and *L. bulgaricus* are quite similar; however, there exist marked physiological differences between them. Strains of true *L. bulgaricus* do not attack maltose, sucrose, and unheated levulose (Kulp and Rettger, 1924) while all strains of true *L. acidophilus* are able to ferment these sugars, producing acid without gas. The first named species will live in the intestine of man and warm-blooded animals when these subjects are given a diet which is high in lactose or dextrin content. On the contrary, under similar conditions *L. bulgaricus* is killed by passage through the digestive tract (Hull and Rettger, 1914; Rahe, 1915; Kulp 1926). Albus and Holm (1926), and, since then, Kopeloff and Beerman (1927), have reported that *L. acidophilus* will grow at lower surface tensions than will *L. bulgaricus*. Day and Gibbs (1928) disagree with the conclusions that these species may be differentiated from each other by surface tension methods, and claim that whatever differences are obtained may be ascribed to varying toxic action.

Unfortunately for the taxonomist and laboratory worker, there are members of this genus which appear to lie midway

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between typical *L. acidophilus* and typical *L. bulgaricus*. The writer has had occasion to study several of these so-called border-line strains which morphologically and culturally are similar to *L. acidophilus* and *L. bulgaricus*, although growth in culture media is more profuse than that of either of these two species. This increased growth constitutes a difference in degree only and, therefore, should not be given much consideration as a differential characteristic. Without exception, the border-line organisms which have been studied by the writer have failed to survive passage through the digestive tract. So-called surface tension experiments classify them with *L. bulgaricus*, leaving small doubt that they are closely related variants of this species.

Within recent years some investigators have designated an organism associated with dental caries and, in rare instances, isolated from intestinal contents, as *L. acidophilus*. Evidence at hand establishes a firm conviction that this organism or group of organisms is wrongly named. Research by Morishita (1928) in our laboratory clearly indicates that the type usually associated with dental caries is not *L. acidophilus* (Moro). The present report does not deal with the dental caries organism or organisms, but mention is made of them here because of their close relation to the subject under discussion and because of the interest they have aroused. Any trained bacteriologist can easily recognize differences between the type of organism commonly associated with caries and representative strains of *L. acidophilus* or *L. bulgaricus*.

Because of the therapeutic properties attributed to *L. acidophilus* by Torrey (1915), Rettger and Cheplin (1921), Kopeloff (1924) and others, the separation of authentic strains of *L. acidophilus* from *L. bulgaricus* types by control laboratories and research workers has assumed much importance, and additional methods of identification appear highly desirable.

In the course of the present investigation it was found that *L. acidophilus* had a decidedly greater tolerance for indol and phenol than did *L. bulgaricus*. A more detailed study revealed that the degree of tolerance could be employed for differential purposes.

Five strains of representative *L. bulgaricus*, two of the maltose-attacking variants of *L. bulgaricus* and five strains of typical *L. acidophilus* were employed in these experiments. A summary of their more important characteristics is given in table 1.

Casein digest made from chemically pure casein containing no carbohydrate was employed as the nitrogenous base for all sugar broths. The inoculum was prepared by centrifuging

TABLE 1

Important characteristics of L. acidophilus and L. bulgaricus strains employed in these experiments

	LACTOSE	MALTOSE	GALACTOSE	UNHEATED LEVULOSE	SUCROSE	INULIN	COLONY TYPE	NO GROWTH AT
								°C.
L. bulgaricus:								
B1	a	0	a	0	0	0	x	20
B2	a	0	a	0	0	0	x	20
B3	a	0	a	0	0	0	x	20
B4	a	0	a	0	0	0	x	20
B6	a	a	a	0	0	0	x	18
B7	a	a	a	0	0	0	x	14
L. acidophilus:								
AH1 (human)	a	a	a	a	a	a	x	20
AH2 (human)	a	a	a	a	a	a	x	20
AH3 (human)	a	a	a	a	a	a	x	20
RH1 (rat)	a	a	a	a	a	a	x and y	20
RH2 (rat)	a	a	a	a	a	a	x and y	20

a = growth and acid production.
0 = no growth.
x and y types of colony (Rettger and Horton, 1914).

forty-eight-hour glucose broth cultures and suspending the sedimented organisms in sterile saline. Broth cultures were incubated for four days at 37°C. Acidity was ascertained by the colorimetric method of pH determination. A decrease of 0.5 on the pH scale lower than the pH of the control mixture (carbohydrate-free casein digest broth and inoculum) was considered as evidence of fermentation. Visible growth was absent in broth

cultures which showed no decrease in the pH. Limiting growth temperature was determined by incubating inoculated glucose broth tubes for seven days.

When levulose is employed in fermentation studies the following precautions should be taken, in order to secure uniform results. Secure the purest sugar obtainable; sterilize a concentrated solution by filtration, and add, aseptically, the necessary amount to each tube of sterilized broth (Kulp and Rettger, 1924).

The X type colony is, in our opinion, a significant characteristic of *L. acidophilus* or *L. bulgaricus*. All isolations of *L. acidophilus* employed in this study were made from typical X type colonies. Strains of true *L. bulgaricus* should produce colonies of a similar type.

Day and Gibbs (1928) state, as their belief, that fermentation reactions cannot be employed to differentiate *L. acidophilus* from *L. bulgaricus*. The writer's results are in disagreement with their claim. He has not been able to secure any culture of typical *L. bulgaricus* which will ferment maltose, sucrose, or levulose. A few strains of border-line organisms have produced acid from maltose, but these types did not attack sucrose or levulose.

Any satisfactory plating medium for *L. acidophilus* or *L. bulgaricus* may be employed in the determination of the tolerance of these species for indol or phenol. Tomato peptone agar (Kulp, 1927) and casein-digest galactose agar (Kulp, 1924) were chosen for these experiments. It appears that the latter is slightly more desirable as a medium for *L. bulgaricus* than the former.

The agar was distributed and sterilized in test tubes, 10 cc. to each tube. The indol was prepared as a 10 per cent alcoholic solution, and the phenol as a 5 per cent aqueous solution; these solutions are self-sterilizing. Cultures in milk which had been incubated at 37°C. for twenty-four hours were employed as the inoculum. Platings containing no indol or phenol were prepared as controls. One cubic centimeter of 1:10,000 dilution of the culture was placed in a sterile Petri dish. The desired amount of indol or phenol solution was added to the melted agar in the tube

and well mixed, after which the agar was added to the culture in the plate. Indol agar plates, phenol agar plates, and the control plates were incubated in separate containers in order to eliminate the effect of volatilized indol or phenol. All cultures were incubated for three days at 37°C. in an atmosphere containing approximately 5 per cent CO_2 (Kulp, 1926).

The inhibitive effect was revealed by the absence of colony development visible to the naked eye. All controls developed an abundant growth.

In later experiments Petri dishes were eliminated. The culture and the desired dilutions of indol and phenol were added to a measured amount of melted agar in test tubes. After mixing thoroughly the agar was allowed to solidify. The cultures thus prepared were placed in tins. After sufficient CO_2 had been added to these tins to constitute about 5 per cent of the atmospheric volume, they were tightly sealed and incubated at 37°C for three days.

Either of the above methods is satisfactory. However, the second is preferable because of its greater simplicity.

The results of representative experiments are indicated in tables 2 and 3.

An experiment was completed which determined the tolerance of several strains of L. acidophilus and L. bulgaricus for given concentrations of indol and phenol over exposure periods of considerable length.

One-tenth of a cubic centimeter of a twenty-four hour culture grown in milk was added to a tube of melted tomato agar (0.4 per cent agar), which was held at 37°C. to prevent solidification. Five-hundredths of a cubic centimeter of a 10 per cent indol solution was mixed with the inoculated agar; similarly 0.5 cc. of a 5 per cent phenol solution was added to another melted and inoculated agar tube. Transfers were made with a 4 mm. loop from each of these cultures into sterile agar at one, two and twenty-four hour intervals. It was discovered that there was a slight decrease in viable organisms of both species after one and two hour exposures. After an exposure of twenty-four hours the L. bulgaricus cultures were found to have been killed; on the other hand, the L. acidophilus cultures contained many viable cells.

TABLE 2

Growth in graded concentrations of indol

STRAIN	1:3300	1:2500	1:2000	1:1650	1:1450	1:1250	1:1100	1:1000	CONTROL
L. bulgaricus:									
B1	+	0	0	0	0	0	0	0	4+
B2	+	0	0	0	0	0	0	0	3+
B3	+	0	0	0	0	0	0	0	2+
B4	+	0	0	0	0	0	0	0	4+
B5	3+	+	0	0	0	0	0	0	4+
B6	+	0	0	0	0	0	0	0	3+
B7	+	0	0	0	0	0	0	0	3+
L. acidophilus:									
AH1	4+	4+	4+	4+	3+	2+	±	0	4+
AH2	4+	4+	4+	4+	4+	3+	+	0	4+
AH3	4+	4+	4+	4+	4+	3+	+	0	4+
AR1	4+	4+	4+	4+	4+	3+	2+	1	4+
AR2	4+	4+	4+	4+	4+	3+	±	±	4+

+ = visible growth.

4+ = very heavy growth.

± = barely visible growth.

TABLE 3

Growth in graded concentrations of phenol

STRAIN	1:750	1:500	1:400	1:300	1:275	1:250	1:225	1:200	CONTROL
L. bulgaricus:									
B1	2+	+	0	0	0	0	0	0	4+
B2	2+	+	0	0	0	0	0	0	3+
B 3	2+	+	0	0	0	0	0	0	2+
B4	2+	+	+	0	0	0	0	0	4+
B5	2+	+	0	0	0	0	0	0	4+
B6	2+	+	+	0	0	0	0	0	3+
B7	2+	+	0	0	0	0	0	0	3+
L. acidophilus:									
AH1	4+	2+	+	+	±	0	0	0	4+
AH2	4+	3+	2+	2+	+	±	0	0	4+
AH3	4+	3+	3+	3+	+	±	0	0	4+
RH1	4+	4+	4+	4+	4+	3+	±	±	4+
RH2	4+	4+	3+	3+	2+	+	+	±	4+

+ = visible growth.

4+ = very heavy growth.

± = barely visible growth.

The results of these experiments indicate that strains of typical *L. acidophilus* can be separated from strains of representative *L. bulgaricus* by the determination of their tolerance for indol or phenol. The very slight tolerance of *L. bulgaricus* for indol and phenol *in vitro* experiments may help to explain why *L. bulgaricus* is not able to survive passage through the digestive tract. Further studies are planned to determine the validity of this theory.

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EFFECT OF HYDROGEN ION CONCENTRATION ON THE TOXICITY OF SODIUM BENZOATE TO MICROÖRGANISMS

W. V. CRUESS AND P. H. RICHERT

Fruit Products Laboratory, University of California

Observations made several years ago in this laboratory indicated that foods of low acidity were much more difficult to preserve with sodium benzoate than those of high acidity. Thus, the spoilage of apple and grape juice by yeast and mold was prevented by 0.1 per cent or less of sodium benzoate, whereas 0.2 per cent failed to prevent growth of bacteria and mold in avocado pulp and in non-acid vegetables stored in weak brine.

It was suspected that hydrogen ion concentration rather than total acidity might be the controlling factor.

A study of the literature showed that the attention of previous investigators had been given to the effect of hydrogen ion concentration on the disinfecting, that is killing action, of sodium benzoate and various other reagents rather than to their preservative action.

However, Herter in 1910 reported that 0.2 per cent sodium benzoate retarded the growth of and gas production by *B. coli* in plain glucose bouillon, but had no noticeable effect in the same medium in the presence of $CaCO_3$. He made no pH determinations and gave the factor of acidity only passing attention.

Barnard (1911) states that benzoic acid is a more effective preservative than sodium benzoate but cites no experimental evidence. Held (1915) found that benzoic acid is less effective as a disinfectant in a medium rich in protein than in one poor in this constituent and states that if the protein binding power of the benzoic acid is satisfied by some other acid, such as tartaric, the concentration of benzoic acid necessary for disinfection is

lessened. Perry and Beal (1920) found that growth of *S. cerevisiae* in glucose bouillon was prevented by 0.5 per cent sodium benzoate and all living cells killed by 3.0 per cent. They also state that benzoic acid is more effective than sodium benzoate in preventing growth of yeasts and molds. Bonacorsi (1923) states that the disinfecting power of several common disinfectants is affected by the pH value of the medium but makes no statement concerning the effect of pH value on their inhibitive action on growth, or activity of microörganisms. Fleischer and Amster (1922) found that the disinfecting power of acid dyes is increased by a decrease in pH value and that of basic dyes by increase in pH value. Kuroda (1926) found that the killing action of sodium benzoate and several other organic preservatives and disinfectants was markedly affected by the pH value of the medium. Disinfection was much less effective in the pH range 5.0 to 8.9 than in that of 1.4 to 3.5. He used *B. coli* and *B. prodigiosus*.

<center>EXPERIMENTAL</center>

Our experiments were conducted for the purpose of definitely determining the effect of hydrogen ion concentration on the inhibiting action of sodium benzoate on the more common food spoilage microörganisms. Those studied were *Saccharomyces ellipsoideus*, isolated from grapes; *S. cerevisiae*; several Mycoderma yeasts; penicillium mold, two species; a mucor mold; a lactic acid culture from E. B. Fred (his culture No. 124-2) vinegar bacteria; *B. coli, B. sporogenes* and *B. subtilis*. It will be impossible to present all of the data in detail in this brief report; instead only the more significant results will be given and for the most part in the form of curves.

For the budding fungi, that is yeasts and molds and for vinegar bacteria, two fruit juice media, grape and apple juices, were used. Different portions were brought to various pH values by the addition of powdered citric acid or N/1 sodium hydroxide or in some instances sodium bicarbonate. These portions of various pH values were subdivided and sodium benzoate added in amounts ranging from no benzoate to an amount at each pH value that it was believed from preliminary tests would prevent growth.
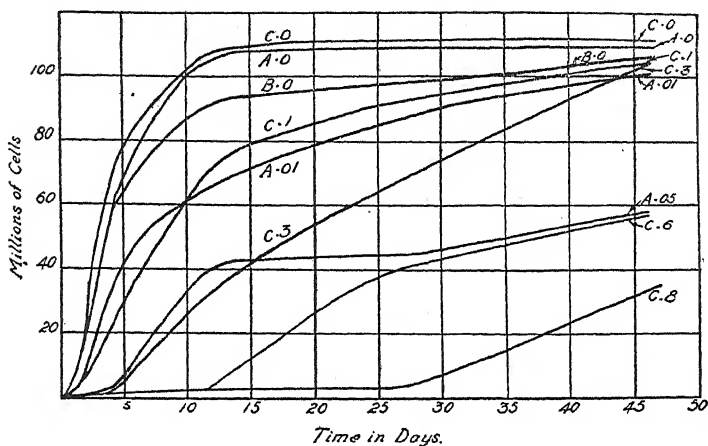
FIG. 1. EFFECT OF pH VALUE ON RETARDING ACTION OF SODIUM BENZOATE ON
MULTIPLICATION OF S. ELLIPSOIDEUS

Curves labeled A 0, A 0.01 etc. represent juice of pH 3.8 and 0, 0.01 etc. gram
benzoate per 100 cc. respectively; B, those of pH 3.0 and C those of pH 6.
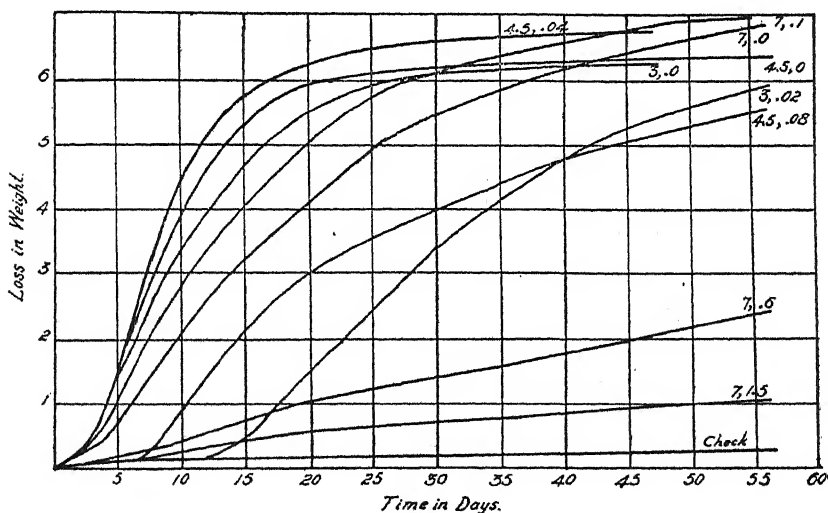
FIG. 2. EFFECT OF pH VALUE ON RETARDING ACTION OF SODIUM BENZOATE ON
FERMENTATION BY S. ELLIPSOIDEUS

The tubed sterilized liquids were inoculated with pure cultures of the microörganisms previously listed. These were stored at room temperature, 18 to 25°C., for six months or longer and observations taken at intervals in order to determine at what
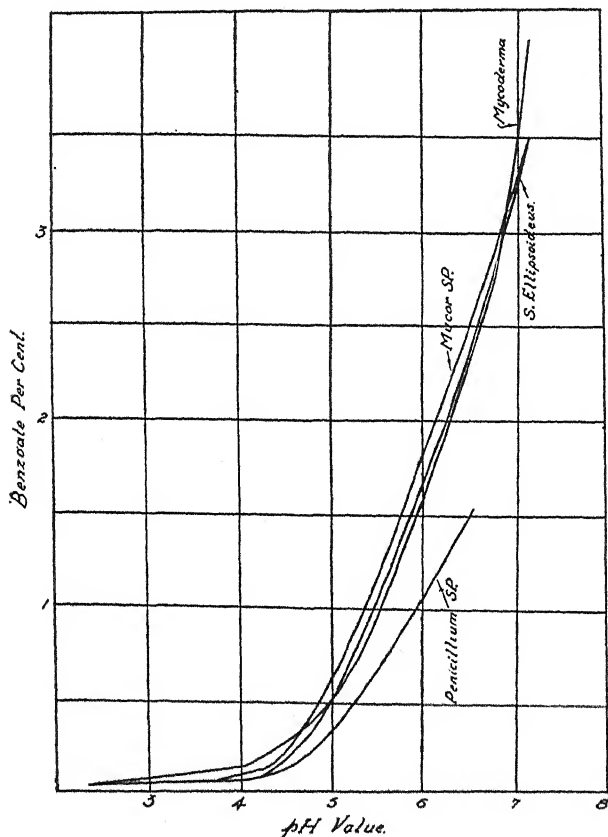
FIG. 3. EFFECT OF pH VALUE ON CONCENTRATION OF SODIUM BENZOATE REQUIRED TO PREVENT GROWTH OF CERTAIN YEASTS AND MOLDS

benzoate concentration in each of the various pH values growth was prevented.

With *S. ellipsoideus* additional observations were taken. In one series of tests the rate of multiplication of the cells at various

benzoate concentrations in juice of three pH values was determined. The results are shown in figure 1. In two other series of tests the rates of fermentation at three and at ten different pH values and various benzoate concentrations were determined. Typical results are given in figure 2.
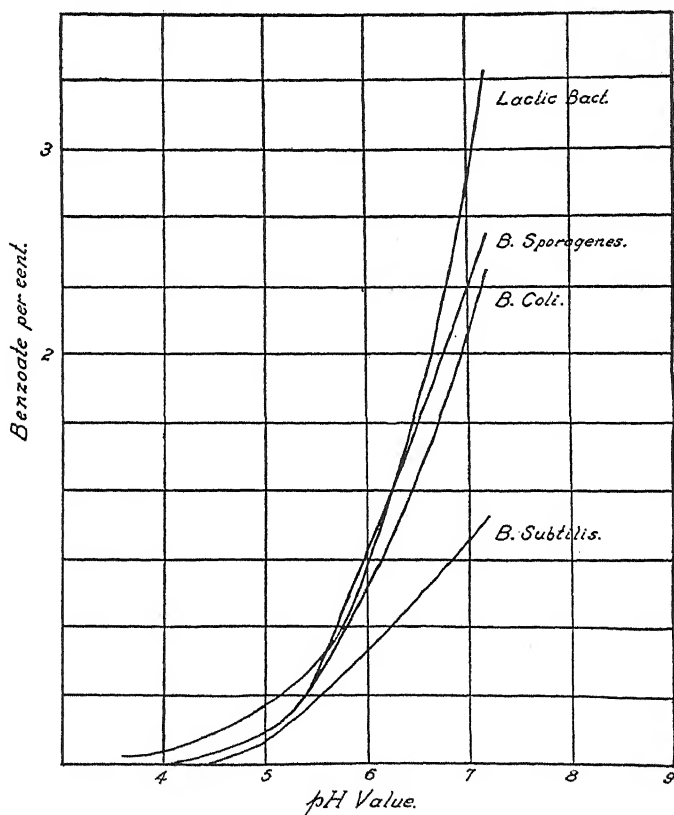
Fig. 4. Effect of pH Value on Concentration of Sodium Benzoate Required to Prevent Growth of Certain Bacteria

B. coli, B. sporogenes, B. subtilis and the lactic culture (E. B. Fred No. 124-2) were grown in a broth of the following composition; bacto pepton 10 grams, glucose 7.5 grams, Libby's extract of beef 10 grams, $MgSO_4$ 0.01 gram, KH_2PO_4 0.25 gram, $(NH_4)_2$

HPO₄ 0.25 gram and water to make 1000 cc. The pH value of portions of the broth was adjusted by the addition of citric acid, N/1 NaOH or powdered NaHCO₃ to values ranging from 3.2 to 10.0 before sterilization. The range after sterilization was 3.6 to 8.7, changes occurring during sterilization being fairly pronounced at the lowest and highest pH values. Benzoate was added before sterilization to subdivisions of the various portions to give a wide range of benzoate concentrations. The tubed sterilized liquids were inoculated with a loopful of the respective cultures. The tubes were incubated a short time, about three days, at 37°C. and were then stored at room temperature 18° to 25°C. for more than six months. To the tubes of lactic and sporogenes cultures was added about ¾ inch of neutral mineral oil.

The benzoate concentrations required to prevent growth of the yeasts and molds at various pH values are given in figure 3; those for the bacteria in figure 4.

Experiments were conducted also with mixed cultures and with ripe olives in brine, avocado pulp, and certain non-acid vegetables. The results were similar to those obtained with pure cultures.

DISCUSSION

The effect of pH value on the inhibiting action of sodium benzoate on the multiplication of *S. ellipsoideus* was very pronounced. In figure 1 curves labeled A represent juice of pH 3.8, those labeled B, pH 3.0 and those labeled C, juice of pH 6.0. At pH 6 the highest concentration of benzoate 0.8 gram per 100 cc. used in this experiment failed to prevent growth, whereas at pH 3.0 growth was prevented by 0.05 gram of benzoate per 100 cc. At pH 6 and 0.8 gram benzoate per 100 cc. growth was delayed for twenty-five days, when slow multiplication ensued. At pH 6 and 0.3 gram benzoate per 100 cc., growth was slow but at 0.1 gram benzoate, growth was only moderately retarded. At the other pH values also, the retarding effect of the benzoate on growth varied with the concentration, although far less benzoate was required to produce a given effect at the lower pH values.

In figure 2 is given the effect of pH value on the retarding action of sodium benzoate on alcoholic fermentation by *S.*

ellipsoideus.   As in its effect on the multiplication of this organism the retarding action of the benzoate was found to be dependent to a very marked degree on the pH value.   The pH values used in this experiment were 3.0, 4.5 and 7.0.   At pH 7.0 fermentation proceeded slowly even in the presence of 1.5 per cent sodium benzoate, the maximum concentration used.   At pH 3.0 fermentation was completely prevented at 0.06 per cent of benzoate.   At pH 4.5 more than 0.1 per cent of benzoate was required to prevent fermentation but 0.04 per cent appeared to stimulate fermentation.   At benzoate concentrations between 0 per cent and the per cent required for prevention of fermentation the retarding action of the benzoate at each pH value in general varied with the concentration of benzoate present.

In figures 3 and 4 it will be seen that the benzoate concentration required to prevent growth varied greatly with the pH value. In the neighborhood of pH 4.5 for the yeasts and molds and at about 5.3 to 5.5 for *B. coli,* subtilis, sporogenes and lactic acid bacteria, there was great change in the slope of the curves.   They became much steeper beyond these points; i.e. much more benzoate is required in the upper range of pH values than in that, say below pH 4.5.

Other data, not shown in the curves, indicate that the vinegar bacteria culture used behaved similarly to the lactic culture; and that at pH values 6.0 to about 9, growth of all organisms used was not prevented by 1.5 per cent of benzoate.   At pH 10, less benzoate was required to prevent growth of one organism than at pH 7.3; in one test 0.7 per cent of benzoate prevented growth of one mycoderma culture at pH 10, but blue mold (a penicillium) grew even in the presence of 1.5 per cent benzoate at pH 10.0.

It is possible that the curves pass through a maximum somewhere between pH 7 and 10 and that the OH^- ion as well as the H^+ ion exerts an effect on the preservative action of sodium benzoate.

It is possible also that some of the inhibitive action of sodium benzoate at pH values in the range 5.0 to 7.0 is due to the Na^+ ion as Winslow (1928) and Doloff (see also the references given by

Winslow in article cited) show that sodium chloride at 0.5 M is slightly toxic in a pepton medium. At pH 7 in our tests concentrations of up to 4 per cent of sodium benzoate were used; giving at the higher percentages concentrations of Na⁺ ions somewhat less than in a .5 M sodium chloride solution.

As would be expected, the pH growth range of the bacteria that are not tolerant to acid (*B. coli*, subtilis and sporogenes) was different from that for the acid tolerant organisms used in these tests and apparently also the turning point of the "pH versus benzoate to prevent growth" curves for these organisms was at a higher pH value than for the acid tolerant organisms. However, the pepton of the medium might have exerted some effect in the tests with these organisms.

It was found that ripe olives and non-acid vegetables in brine and avocado pulp inoculated with mixed cultures required many times more benzoate for preservation at pH values above 5 than at pH 3.5 to 4.0. One-tenth of 1 per cent benzoate had no apparent retarding effect on the spoilage of these products unless acid was added. It is therefore extremely dangerous to attempt to preserve such non-acid products with sodium benzoate unless acid is added. *B. subtilis* was considerably less tolerant to the acid and benzoate than *B. coli* and sporogenes.

SUMMARY

1. The retarding action of sodium benzoate on the rate of multiplication of *S. ellipsoideus* is much stronger at pH values of 2.5 to 4.5 than at 5.0 to 9.

2. A similar relationship holds for the retarding effect of sodium benzoate on alcoholic fermentation by this microörganism.

3. The concentrations of benzoate required to prevent the growth of *S. ellipsoideus*, *S. cerevisiae*, a mucor mold, two penicillium molds, three strains of mycoderma yeast, a lactic acid bacterium, a vinegar bacterium, *B. coli*, *B. subtilis* and *B. sporogenes* was greatly affected by the pH value of the medium. Much more benzoate was required at pH values near neutrality, e.g. pH 5 to 8 than at those in the moderately acid range 2.5 to 4.5.

4. While these observations are primarily of scientific interest

they also have an important bearing on the preservation of non-acid foods such as ripe olives, avocado pulp and non-acid vegetables by sodium benzoate.

5. Preliminary experiments have proved that similar relationships hold for certain other food preservatives.

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THE DESTRUCTION OF LUMINOUS BACTERIA BY HIGH FREQUENCY SOUND WAVES

E. NEWTON HARVEY AND ALFRED L. LOOMIS

From the Loomis Laboratory, Tuxedo Park, New York, and the Physiological Laboratory, Princeton University

In a previous communication (1928) we have described the dimming of the light of luminous bacteria exposed to high power high frequency sound waves, generated by the apparatus of Wood and Loomis (1927). This consists of a 2-kilowatt oscillator giving a maximum of 50,000 volts across a quartz crystal in an oil bath. A test tube containing the bacteria suspended in sea water touches the surface of the oil, and the sound waves generated in the vibrating quartz crystal by the piezo-electric effect, pass through the oil, the glass of the test tube and the sea water suspension of the bacteria. Considerable energy is introduced, which heats the media, so that in the original experiments, using a frequency of 406,000, the bacteria were first cooled to 1.5° and rayed until their temperature reached 21.5°. This takes only a few minutes, but in this time the bacterial light is perceptibly dimmed, although not extinguished.

If a low powered oscillator is used and the bacteria mounted under a cover slip on the crystal and placed on the microscope stage without an oil bath, as described in our previous paper (1928), one can observe great agitation of the bacteria but no breaking up, such as happens to infusoria. The bacteria are carried into nodes of a complex wave pattern and remain unharmed, but the convection currents in a large test tube prevent the formation of permanent nodal accumulations of the bacteria.

In our recent experiments the bacteria (rod shaped *Bacillus Fisheri*)[1] were placed in a large test tube containing a glass coil

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through which ice water was circulating to keep down the temperature, and with another glass tube opening under the surface of the bacterial suspension, through which air, filtered through sterile cotton wool, was bubbled to keep the suspension stirred. The top of the test tube was cotton plugged and sterilized and the whole experiment so carried out as to prevent contamination with foreign bacteria. The high power oscillator was used with a frequency of 375,000 per second. It was found that even with ice water cooling of the suspension, the oil bath became so hot that in thirty minutes the temperature of the bacteria had risen to 35°C. As this is sufficient to cause near extinction of the light of the bacteria without exposure to sound waves, the oil bath in later experiments was also cooled with coils of lead tubing. The lead tubing and all metal parts near the high tension field must be grounded to prevent sparking.

After the exposure to sound waves the bacteria were inoculated on agar slants and Petri dishes to determine if all were killed. A number of experiments were carried out, as follows:

A1. Rayed for thirty minutes, when the suspension had almost completely cleared, an indication that the bacteria are broken up, and the light had disappeared, but this might be due to heating, as the temperature had risen to 35°C.

A2. Same procedure and results as A1.

A3. The same bacterial emulsion was heated to 35°C. in a water bath for thirty minutes and then cooled. It did not clear, but the bacterial luminescence had disappeared.

Platinum loop transfers of A1, A2, and A3, were made to agar slants as well as from a control suspension of bacteria unheated. After sixteen hours the control showed good luminescence and growth while A1, A2 and A3 showed no luminescence or growth. After forty-eight hours, A3, the heated tube, showed good luminescence from about one hundred colonies while A1 and A2 showed luminescence and growth from twelve and three colonies respectively. Evidently the heating had seriously injured the bacteria and the raying plus the heating had killed nearly all. There were no growing colonies without luminescence. This experiment indicates that the bacteria are mostly killed by one

half hour raying, but does not tell whether the luminescence can be completely extinguished, as the temperature rose too much.

In another experiment (B1) the bacteria were rayed for an hour and the temperature kept below 19°C. by cooling the oil as well as the bacterial suspension. The rayed bacteria showed no growth or luminescence when plated on the Petri dishes or when inoculated on agar slants, while the control inoculations grew and luminesced brilliantly. The luminescence dims rather quickly on raying but the last trace of light persists for something like twenty to thirty minutes.

Examined under the microscope the rayed suspensions show practically no bacteria while the controls are full of actively moving individuals. As indicated by the clearing of the suspensions the bacteria must be thoroughly torn up and cytolysed as they are when placed in water. The silkiness, due to optical effects of needle particles, observed when normal luminous bacteria suspended in sea water are gently agitated, had disappeared in the rayed tubes. Another experiment (B2), like the above, but in which the temperature was kept below 15°C., gave identical results.

C1. Finally a very dense emulsion of luminous bacteria in sea water was rayed for ninety minutes and loop samples inoculated on agar slants every five minutes. The temperature was kept below 16°C. throughout the experiment. The luminescence gradually dimmed until it was faint (forty-five minutes) and only a just perceptible luminescence remained after ninety minutes raying. After fourteen hours the inoculations made up to fifty minutes raying all showed good luminescent growth, the fifty-, sixty-, and seventy-minute inoculations a few colonies, and the eighty-, and ninety-minute inoculations no luminescence. After twenty-four hours all inoculations showed good luminescent growth except the ninety-minute with one colony and after thirty-six hours, all inoculations showed some luminescent growth. The raying had evidently killed most of the bacteria, only a few more resistant ones persisting with their power of growth retarded.

The rayed emulsion had partially cleared and lost most of its silky appearance on gentle shaking, characteristic of rod and

needle particles in suspension and examined under the microscope showed very few rods but many small granules, like fragments of bacteria. There is also a coagulum of fiber-like material which collects on the side of the test tube and cooling tubes, in all the rayed suspensions. This is not cotton fiber as it gives a strong xanthoproteic test and is undoubtedly composed of the debris of the bacteria stuck together in some peculiar manner under the influence of the sound waves. It never appeared in control unrayed tubes of bacterial suspension through which air was bubbled as in the experimental tubes.

It should again be emphasized that the conditions of the raying are such in these experiments, namely, a round bottomed tube and constant agitation, that no standing waves can be set up and consequently the bacteria cannot be thrown into nodes, where they remain unharmed, as happens in capillary tubes or spaces.

In order to see whether needle crystals could be broken up by raying, a suspension of benzopurpurin in water was rayed for one hour, with ice water cooling. The needle crystals, easily visible under high power of the microscope, are broken apart into shorter lengths but not sufficiently so as to abolish the silkiness normally exhibited by these solutions. A silky soap solution with needle crystals was not affected by one hour's raying. Perhaps we should not expect such needle particles whose consistency and rigidity undoubtedly differ from that of bacteria to behave in the same way.

In conclusion we can state that, under proper conditions of raying, luminous bacteria can be broken up and killed by sound waves of approximately 400,000 frequency and the solutions sterilized, but that the method is not one of any practical or commercial importance because of the expense of the process.

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AN IMPROVED METHOD OF CULTURE FROM A SINGLE BACTERIAL CELL

SYDNEY G. PAINE AND J. C. RAMCHANDANI

Department of Plant Pathology, Imperial College of Science and Technology, London

The method to be described is a modification of the one given by the senior writer in an earlier number of this JOURNAL (Paine, 1927) coupled with that of Burri (1909). Attenuations from the culture are made in five sterile watch glasses, as in the method of Paine but, in place of sterile water, use is made of a sterile solution of nigrosine as in the method of Burri. With the precautions described in the earlier paper the appropriate dilutions are taken up with a steel mapping-pen and, instead of being spotted on the surface of agar in a dish, are drawn in lines on the surface of a film of agar previously prepared on a long narrow coverslip. In order to prevent ploughing up the surface of the agar the steel pen is attached to its holder by a short coil of spring-brass wire. In this way sufficient rigidity for the control of the pen's direction can be retained while allowing only the slightest pressure of the point of the pen on the surface of the agar. The coverslip is mounted above a glass slide, film side down, conveniently supported at the ends on thin strips of cardboard in such a way that contact of the agar film with the glass slide is just prevented. The ink lines are examined under the 12th inch objective and the positions of what appear to be single bacterial cells are marked down by spots of Chinese ink. The slides are then placed in a moist chamber and incubated over-night. Any colonies which develop at the marked spots from single cells will be of approximately the same size and may safely be assumed to be pure, while any which develop into larger that minimal size may be discarded as impure.

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THE VALUE OF VEGETABLE EXTRACTS IN CULTURE MEDIUMS[1]

LUTHER THOMPSON

Section on Clinical Pathology, The Mayo Clinic, Rochester, Minnesota

A survey of the literature dealing with the use of various extracts from vegetables and meats as enriching agents in culture mediums shows that certain workers (Davis, 1917 and 1921; Funk and Dubin, 1921; Hosoya and Kuroya, 1923a and b; Kligler, 1919; Lloyd, 1916–17; Thjötta, 1921; Thjötta and Avery, 1921 a and b; Uyei, 1927) have considered that they were dealing with vitamines or growth-accessory substances for bacteria. The organisms which are grown with more than average difficulty are the ones usually spoken of as requiring the presence of vitamines, such as *Diplococcus pneumoniae, Neisseria intracellularis, Neisseria gonorrhoeae, Hemophilus influenzae* and various streptococci. Pacini and Russell (1918) have shown that *Eberthella typhi*, when grown in a synthetic medium, will furnish vitamines which will maintain normal development in rats, but the work of others (Ayers and Mudge, 1922; McLeod and Wyon, 1921; Thjötta, 1924) indicates that the vitamines A and B, effective in animal nutrition, do not influence bacterial growth. Another group of investigators (Cole and Lloyd, 1916–17; Morgan and Avery, 1923; Mueller, 1922 a and b; Rivers and Poole, 1921) has called attention to the value of extracts of meat, blood and various vegetables in the culture of bacteria without suggesting anything regarding the nature of the growth-promoting substance.

Extracts were made of potato, carrot, radish, spinach and beef heart, the latter for comparison. One hundred fifty grams of grated or ground material were added to 200 cc. of distilled

[1] Read before the Society of American Bacteriologists, Richmond, Virginia, December 27 to 29, 1928.

water.   The mixture was stirred for ten minutes and filtered through cheese cloth to remove the coarser particles.   The filtrate was then centrifuged at high speed for thirty minutes and the supernatant fluid filtered through Seitz filters and stored in sterile test tubes in the ice box.   Table 1 shows the total nitrogen and reducing sugars in these extracts, and indicates approximately what may be expected with this method of preparation, although there are variations between different lots.

When freshly made, the various extracts seemed to be about equal in growth-promoting power, but certain ones, especially those from spinach and beef heart, turned acid on standing and a precipitate was formed, after which their value was greatly decreased.   The various extracts were heated to 56°C. for thirty

TABLE 1

EXTRACT	REDUCING SUGAR	TOTAL N
	per cent	*mgm. per cc.*
Potato..	0.21	1.23
Carrot..	0.86	0.42
Radish..	0.36	0.18
Spinach...	0.12	0.42
Beef heart...	0.04	0.68

minutes, at 100°C. for ten minutes and run in the autoclave at 20 pounds pressure for fifteen minutes.   After heating they were tested for growth-promoting power.   In general it was found that heat had little effect except as it caused precipitation of the protein content.   This was marked in some of the extracts. Potato extract was precipitated least and was found to be the best of those tried, so that most of the following work was done with it.   This extract seems to keep for three to four months without losing effectiveness.   In order to learn whether all the growth-giving material could be removed by precipitating the protein, a sample of potato extract was treated with 9 volumes of tungstic acid.   The resulting precipitate was removed by centrifuging and the supernatant fluid was filtered to insure sterility. Tests showed that approximately half the total nitrogen was

proteid nitrogen.   The precipitate was dissolved in weak alkali and made up to original volume.   By using volumes of the two fractions which were equivalent to the original, it was found that both the proteid nitrogen and nonproteid nitrogen of potato extract would support equal growth of test organisms, but the growth was less than with the original.   In other words, it was found that within certain limits, growth corresponded to the nitrogen content of the extract.

By the addition of increasing amounts of the extracts to tubes each containing 6 to 7 cc. of plain broth, which in this work was used as a routine basic medium, it was found that as little as 0.01 cc. would allow growth of many strains of streptococci which did not grow in the basic medium.   An increase in the

TABLE 2

	TUBE 1*	TUBE 2*	TUBE 3*	TUBE 4*	TUBE 5*	TUBE 6*	TUBE 7*	TUBE 8*	TUBE 9*
Potato extract added, cc..	0.01	0.02	0.03	0.05	0.1	0.2	0.5	1.0	None
Growth in twenty-four hours.................	+	++	++	+++	+++	+++	+++	+++	None

* Each with 6 cc. of broth.

luxuriance of growth of streptococci was not noted when amounts greater than 0.2 cc. were added to the same amount of medium. Table 2 shows a typical result.

After the addition of the extract all tubes were incubated for twenty-four hours as a proof of sterility before they were inoculated.   Then one capillary drop of a light suspension of hemolytic streptococcus in physiologic sodium chloride solution was added to each tube.

The value of potato extract in promoting growth of streptococci was tested as follows: To tubes containing 6 to 7 cc. of nutrient broth was added 0.2 cc. of the extract.   The streptococci were picked from blood agar plates and were kept as stock cultures on blood agar slants.   From these stock cultures a small amount was transferred to the test medium with a straight wire, care being taken not to carry over any of the medium.   Nineteen

cultures of hemolytic streptococci grew freely within twenty-four hours. Four cultures of *Streptococcus viridans* were tested, three from the blood in cases of subacute bacterial endocarditis and one from chest fluid. All grew well in twenty-four hours, with the exception of one of the strains from endocarditis, which showed fair growth in forty-eight hours.

One culture of a hemophilic organism, corresponding culturally and morphologically with *Hemophilus influenzae*, did not grow in nutrient broth and potato extract, but did grow in brain broth and potato extract. Brain broth alone did not support growth of this organism.

Potato extract, when added to blood agar, has given a somewhat more vigorous growth of *Neisseria gonorrhoeae* than was obtained without it, but the results are not as striking as with the other types of organisms mentioned.

Certain experiments have been made to test the suitability of potato extract as an enriching substance in sugar fermentation tubes by adding 0.1 cc. of the extract to each tube containing 5 cc. of the sugar broth used (nutrient broth and 0.5 per cent sugar). The typical fermentation reactions of *Eberthella typhi* were unchanged by the addition of the extract. As a further test two strains of streptococci were chosen, one *Streptococcus viridans* which grew in the sugar broths without the addition of any enriching fluid, and the other a hemolytic streptococcus which would not. With each organism two series of tubes were set up, the first series with the extract added and the second series without it. The *Streptococcus viridans* fermented identical sugars in each series, thus proving that the addition of extract did not affect the results. The hemolytic streptococcus fermented certain of the sugars in the series of tubes containing the potato extract and failed to grow in the other series.

Attempts to grow *Diplococcus pneumoniae* in nutrient broth and potato extract have shown that growth may be obtained by using more of the extract than seems necessary with streptococci. The growth so obtained in two instances was used as an antigen in the typing test with clear-cut results, while the brain broth

cultures did not agglutinate.   Further attempts are being made to determine the value of potato extract for this purpose.

The growth-promoting substance in vegetable extracts has been called a vitamine or growth-accessory factor by most workers.   As previously stated, some have found that vitamine B does not aid the growth of bacteria, and have suggested that a special vitamine is necessary for bacteria.   It is difficult to devise experiments which will show conclusively that a substance of unknown composition is or is not a vitamine without resorting to well established experiments in animal nutrition.   I believe that the substance in fresh plant and animal tissues, which are water-soluble, may be better considered as food substances for bacteria than as food-accessory factors or vitamines.   The following experiments and observations are made in support of this statement.

A tube of potato extract was used as a medium for growing *Salmonella schottmülleri*.   After seven days' incubation the culture was heated to 80°C. for ten minutes and centrifuged at high speed to throw down the bacteria.   The clear supernatant fluid was added to tubes of plain broth in varying amounts up to 0.2 cc.   After these tubes were incubated to prove sterility they were inoculated with a culture of hemolytic streptococcus which was known to grow well when 0.2 cc. of potato extract was used. Growth was not obtained after the addition of the extract which had been used as a medium for *Salmonella schottmülleri*.   Evidently the growth-promoting material had been exhausted by the paratyphoid organism.   It is not surprising that a medium containing such small amounts of nitrogenous and carbohydrate substances should be exhausted by an actively growing organism in seven days.   This experiment indicates that the growth-promoting substance is a food rather than a food-accessory factor, and it also shows that the growth of *Salmonella schottmülleri* of itself did not produce a food-accessory factor for the streptococcus used.

When the foregoing experiment was repeated with one change only, that is, the substitution of plain broth for potato extract, growth of the streptococcus was obtained.   The paratyphoid

organism evidently does not use up all of the nitrogenous material when as much as 1 per cent peptone is supplied, but it does exhaust the nitrogenous material in potato extract if only 1 to 2 mgm. for each cubic centimeter is present. As a further test a plain broth containing 0.1 per cent peptone was used instead of the usual 1 per cent and the experiment was repeated. It was found that, with the smaller amount of peptone, growth of the streptococcus did not occur when the supernatant fluid of the paratyphoid culture was added to plain broth as before. To make sure that this was not a chance result the experiment was repeated several times, using six different Gram-negative bacilli for the treatment of the 1 and 0.1 per cent peptone broths. The cultures so obtained were tested for power to stimulate growth of ten different cultures of streptococci, with the results mentioned. It is understood, of course, that the streptococci used did not grow perceptibly in nutrient broth with the rather light inoculations employed. From these experiments it appears that, when a larger amount of nitrogenous material is used, the growth of the Gram-negative bacilli comes to an end before the food is exhausted and that the residual end-products furnish a suitable pabulum for the growth of streptococci. But when a small amount of nitrogenous material is employed, as in 0.1 per cent peptone or in potato extract, the residual material is not sufficient. This is taken as evidence that the significant factor in accelerating growth of streptococci is proper food rather than the presence of food-accessory factors.

In regard to the effect of the carbohydrate in the various extracts used, it was noted that there was no correlation between the amount of reducing sugar present and the power to stimulate growth. Extract of beef heart which contained only 0.04 per cent was slightly superior to carrot extract which contained twenty times that amount of reducing sugar.

SUMMARY

Aqueous extracts of potato, carrot, spinach, radish and beef heart were sterilized by filtration and added to nutrient broth aseptically. It was found that as little as 0.01 cc. of these

extracts, when added to 6 to 7 cc. of nutrient broth, would promote growth of many streptococci which did not grow in the broth alone, while 0.2 cc. was sufficient to give vigorous growth of most streptococci.   Potato extract was found to be the most satisfactory, because of its high nutritive value and because it keeps well without change in reaction or precipitation of protein.

Heat was found to have an effect on the extract which was proportional to the precipitation, those media giving the most precipitate being the least useful in accelerating growth.

Both the proteid and nonproteid nitrogen fractions of the potato extract serve to stimulate growth of streptococci, but not in as marked a degree as the whole extract.

Potato extract may be used in place of fresh blood in many instances where it is not essential to observe hemolysis.   It is helpful in getting a growth of streptococci and pneumococci free from cells and precipitated fractions of medium.   As an enriching substance in ordinary fermentation tubes it allows growth of streptococci without interfering with the action on the sugar.

The substance in potato extract responsible for promoting growth is thought to be nitrogenous material which furnishes suitable food for the bacteria rather than food-accessory substances.

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STRAIN VARIATIONS IN C. BIFERMENTANS[1]

E. T. DRAKE AND W. S. STURGES

Laboratory of the Cudahy Packing Company, Omaha

Clostridium bifermentans was first isolated and described by Tissier and Martelly (1902), and was generally accepted as a distinct and definite species until Hall (1922) observed that some bifermentans strains were motile, therein differing from the original description. Correlated with this difference in motility, he found certain other differences—in agglutination and in deep colony morphology. He proposed dividing this species into two species, retaining the name *C. bifermentans* for the non-motile strains, and designating the motile strains as *C. centrosporogenes*.

Kahn (1924), after an extensive comparative study of the anaerobes, recognized this designation, but suggested that perhaps it should be regarded as "a strain variant of *C. sporogenes* and that the cultural features exhibited by Hall's type are not sufficient to warrant a new specific name." Similarly Weinberg and Ginsbourg (1927), in a review of proposed new species, state that *C. centrosporogenes* is merely a particular strain (souche particulière) of *C. sporogenes*.

Sturges and Drake (1928), however, examining Hall's strains of *C. bifermentans* and *C. centrosporogenes* in sealed capillary tubes, found motility in all strains examined, and suggested that *C. centrosporogenes* was probably much more closely related to *C. bifermentans* than to *C. sporogenes*.

The proteolytic ability of *C. bifermentans* is another point on which the results of different investigators vary. Tissier and Martelly (1902), Hall (1922), Reddish and Rettger (1924) and

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Weinberg and Ginsbourg (1927) considered this species as strongly proteolytic. Kendall, Day, and Walker (1922) found moderate proteolysis, and Kahn (1924) reported that proteolysis was "definitely mild in type."

This reported variance in the proteolysis of *C. bifermentans* (which name in some cases undoubtedly included *C. centrosporogenes* since some of the results were reported before Hall's proposed division of the species) was readily accounted for when a preliminary investigation was made of the proteolytic ability of one of Hall's bifermentans and one of his centrosporogenes strains. The comparatively slow action of the bifermentans strain suggested that Hall's division of the group might be substantiated by another and a more definite criterion than aerobic motility tests.

It was further planned to study fermentation reactions, morphology, colony form, and other possible indications of a logical grouping. To show the similarities and dissimilarities of *C. sporogenes* to the *C. bifermentans* group, the former organism was included in all tests.

EXPERIMENTAL

Strains used

The strains used in this study were:

1. *C. bifermentans**..................................Lister Institute
2. *C. bifermentans*...................................Hall 50
3. *C. bifermentans*...................................Hall 70
4. *C. bifermentans*...................................Hall 258
5. *C. bifermentans*...................................Hall 408 C
6. *C. centrosporogenes*.............................Hall 76
7. *C. centrosporogenes*.............................Hall 262
8. *C. centrosporogenes*.............................Hall 472
9. *C. centrosporogenes*.............................Hall 516
10 to 21 inclusive. Strains isolated in this laboratory:
    C. sporogenes...................................Hall 52
    C. sporogenes...................................Hall 54

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* It should be noted that this strain was named before a division into bifermentans and centrosporogenes had been suggested.

Purity of these strains was insured by plating and by repeated examinations. In the experimental work, every culture was examined for contamination.

Morphology

The most consistent and convenient observations on morphology were made on a minced pork medium, buffered to pH 8.0 with sodium phosphate. Cultures were grown at 37°C., and Gram stains made. Measurements are given for a typical strain (no. 1) in table 1. The reason for a departure from the usual

TABLE 1

Measurements of strain 1

	WIDTH			LENGTH		
	Minimum	Majority	Maximum	Minimum	Majority	Maximum
	μ	μ	μ	μ	μ	μ
Vegetative rods:						
16 hours......................	0.5	0.7	1.1	1.3	2.5	7.0
40 hours......................	0.5	0.9	1.6	1.0	4.0	6.5
3 days......................	1.2	1.4	1.6	2.2	4.5	6.0
7 days......................	0.9	1.0	1.1	4.0	8.0	260.0
Sporangia:						
All ages......................	0.7	0.8	1.1	1.8	2.5	3.5
Free spores:						
All ages......................	0.6	0.8	0.9	1.0	1.4	2.2

custom of reporting measurements in a few figures is obvious. Almost all rods and sporangia are Gram-positive, regardless of age. Spores swell the rods slightly, but definitely, in most of the sporangia. Apparently about 95 per cent of the cells form spores.

While most strains rarely show more than three or four rods in a chain, strains 9, 12, 13 and 14 often have as many as twenty in a chain. In strains 9 and 14, these chains break up as the sporangia develop, but in strains 12 and 13 long chains of mature sporangia are seen. Strains 2, 3, 5, and 7 show little, if any, growth before twenty hours. Vegetative rods measure about the same as strain 1, but sporangia are larger, measuring 0.9 to 1.8μ by 2.5 to 5.0μ. Sporangia of strains 12 and 13 are inter-

mediate in width, this measurement varying from 1.2 to 1.4μ. Strain 8 is exceptional in that the spores rarely swell the rod.

C. sporogenes, under the same conditions, measures 0.7 to 0.9μ by 3 to 5μ at sixteen hours, and 0.5 to 0.7μ by 1 to 4μ at forty hours. After forty hours most of the cells become Gram-negative. Less than 25 per cent of the cells form spores. The spores swell the rods markedly.

Motility was demonstrated for all these 21 strains by examination in sealed capillary tubes. Examined under cover slips, young cultures of strains 1, 5, 6, and 8 to 21 usually, but not always, showed a few motile cells. Motility was but rarely demonstrated by this method for strains 2, 3, 4, and 7. *C. sporogenes* cultures usually contain a large number of motile cells, regardless of the method of examination or age of culture.

Colony form

Thompson (1926) claimed to be able to differentiate between *C. bifermentans* and *C. centrosporogenes* by the types of colonies formed on blood agar. Accordingly, dilution plates were poured, using a veal infusion agar (pH 7.5). To each plate, 0.5 cc. of fresh sterile defibrinated beef blood was added before the agar was poured. These plates were incubated in an atmosphere of hydrogen at 37°C. for forty-eight hours.

Under these conditions, deep colonies develop to a diameter of from 0.1 to 1.0 mm. Several types of colonies may be observed; ameboid masses, containing irregular granules of varying size and density; colonies of simple lens shape; dense lobate colonies appearing to be aggregates of 5 to 20 lens-shaped lobes; and irregular masses with small bud-like protuberances. Crowded plates show only the first type of colony. Edges of colonies may be either entire or irregular to filamentous.

A zone of hemolysis surrounds each colony. Strains 2, 3, 4 and 7 produce a narrow zone of hemolysis, while the other strains produce a comparatively wide zone—as wide or wider than the diameter of the colony. The hemolytic zone of *C. sporogenes* is several times as wide as the diameter of its colony.

Viscosity

Kahn (1924) mentioned a "mucoid-like deposit" in cultures of *C. centrosporogenes*. We have made similar observations in bouillon, gelatin, and meat media. Strain 1, alone, consistently shows this property. In general 2, 3, 4, 7, 12 and 13 usually show a minimum of ropiness or none at all, while the other strains usually show a considerable amount. *C. sporogenes* never produces any viscosity.

Correlated with this property of viscosity is the occurrence of a clear zone in the upper part of gelatin and broth cultures.

TABLE 2

Acid production from carbohydrates

	GLUCOSE	LEVULOSE	GALACTOSE	MANNOSE	MALTOSE	GLYCEROL	SORBITOL
Strains 1, 5, 8 to 12 inclusive..	+	+	0	+	+	+	+
Strains 2, 3, 4, 6 and 7.......	+	+	0	+	+	+	0
C. sporogenes 52 and 54......	+	+	Slight	0	+	Slight	+

Fermentations

A basic medium containing 1 per cent peptone and 0.3 per cent beef extract was used. To this was added 1 per cent of the carbohydrate. The cultures were incubated three days in hydrogen at 37°C. The pH of the medium before inoculation was between 7.0 and 7.5. Inoculations were made from cultures grown for twenty-four hours in the basic medium. The carbohydrates fermented are shown in table 2. Gas production is variable in the bifermentans group except that it is almost always produced from glucose, maltose and mannose.

Protein metabolism

Observations of the digestive action of these strains on gelatin, coagulated egg white and coagulated blood serum media showed slow liquefaction of these substrates by strains 2, 3, 4 and 7. Digestion by the other strains was more rapid and more extensive. *C. sporogenes* liquefied these media rapidly after a short lag period.

Gelatin was selected as the most suitable medium for obtaining quantitative data for possible differentiation. Progress of proteolysis was measured in terms of electrical conductivity change in the medium. The relation between the production of ammonia, which is an end product of protein metabolism, and con-

TABLE 3

Conductivity change in gelatin

Expressed as reciprocal ohms $\times$ 10^3

STRAIN	20 HOURS	44 HOURS	4 DAYS	11 DAYS	51 DAYS
1	3.2	7.0	11.7	14.9	18.3
2	1.1	2.1	4.1	9.2	14.9
3	0.4	1.1	3.1	7.3	13.3
4	0.5	1.5	3.7	7.5	11.0
5	3.4	7.9	11.5	15.2	19.4
6	2.5	5.3	10.6	16.8	20.1
7	1.2	3.1	5.4	9.8	12.2
8	2.3	6.3	10.6	14.0	18.8
9	2.2	5.1	8.8	13.9	21.4
10	3.3	5.5	7.4	12.0	18.4
11	2.6	6.6	11.1	15.1	19.4
12	1.9	3.6	5.5	8.9	18.5
13	1.9	3.5	5.3	8.6	20.0
14	2.2	4.4	7.9	13.1	22.3
15	2.5	4.5	8.2	15.1	19.6
16	2.0	4.4	8.1	12.9	18.7
17	1.8	3.5	6.4	12.0	20.4
18	1.8	3.6	6.6	10.6	20.8
19	2.0	5.2	10.1	13.7	20.4
20	3.8	8.9	14.4	16.8	21.8
21	2.9	5.6	10.2	13.4	17.3
C. sporogenes 52	0.2	13.4	20.0	24.4	28.0
C. sporogenes 54	0.5	12.2	18.6	21.9	26.2

ductivity change has been discussed and a technique for the purpose described by Parsons and Sturges (1926).

Nutrient gelatin was inoculated from twenty-four-hour cultures of each strain, and incubated in an atmosphere of hydrogen at 37°C. Resistances of cultures were read at twenty hours, forty-four hours, four days, eleven days, and fifty-one days.

Ten tubes were inoculated with each strain, and duplicate tubes were used at each interval. Readings were made at 30°C.

From the resistance, conductivity was computed, and the similarly determined conductivity of the uninoculated controls was subtracted. Conductivity change is expressed in reciprocal ohms $\times 10^3$. For the sake of brevity and clarity, and since the readings on duplicate tubes showed but few deviations of more than 2 per cent from the average, only the averages are reported in table 3.

DISCUSSION

The data on morphology show several points of difference between *C. sporogenes* and all the other strains. The former is more persistent in its motility; its spores swell the rods more; vegetative rods of *C. sporogenes* cultures become smaller as the cultures mature, while rods in cultures of the bifermentans group become larger; the majority of the rods in *C. sporogenes* cultures lose their property of retaining the Gram stain, decrease in size and die without sporulating, while most of the organisms in cultures of the bifermentans group produce spores.

Several possible groupings of the 21 strains are suggested by the data. Number 8 differs in the fact that its spore does not swell the rod. Strains 9, 12, 13 and 14 show more chain formation, 12 and 13 being more persistent in this respect. Strains 2, 3, 4 and 7 differ in the slowness of their growth, the sensitiveness of their motility, and the width of their sporangia. Strains 12 and 13 are intermediate with regard to sporangium width.

As to colony formation, no consistent differences are observed, except in the ratio of the diameter of the colony to the width of the zone of hemolysis. *C. sporogenes* is distinctly more hemolytic. Strains 2, 3, 4 and 7 are feebly hemolytic. The other strains are intermediate.

The production of viscosity again distinguishes *C. sporogenes* from the bifermentans group. It also gives an indication, although an undependable one, of a differentiation between strains 2, 3, 4, 7, 12 and 13, and the remainder of the group.

The fermentation of mannose furnishes a definite and precise

differentiation of *C. sporogenes* from the bifermentans group. The reactions in galactose and glycerol, although less definite, appear to be constant. The fermentation of sorbitol demarcates strains 2, 3, 4, 6 and 7 from the remainder of this group.
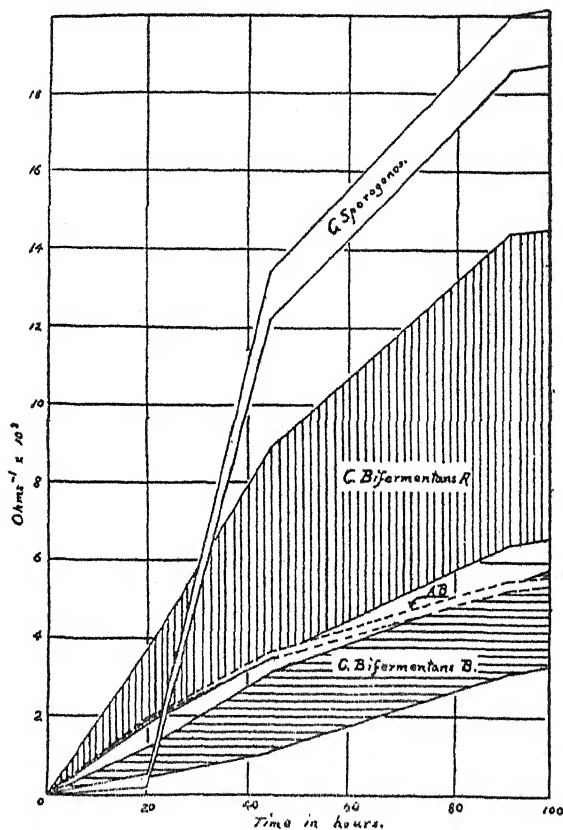
FIG. 1. RANGE OF CONDUCTIVITY CHANGE IN GELATIN, ALL STRAINS—EARLY AGES

The data on digestion of gelatin in table 3 suggest no basis for a division of the bifermentans strains. However, in figures 1 and 2 we have divided these strains into groups on the basis of previously described differences in morphology, hemolysis, viscosity and macroscopic observations of liquefaction of proteins.

The highest and the lowest values (conductivity change) for each day in each group are plotted as points.   The areas between the lines connecting these points will be referred to as zones. The zoning arrived at in this manner gives some support to these other differentiations as shown by the separation of the zone designated *A* from that designated *B*.

Strains 12 and 13, however, exhibit an intermediate type of activity as was the case with their other properties.   The course
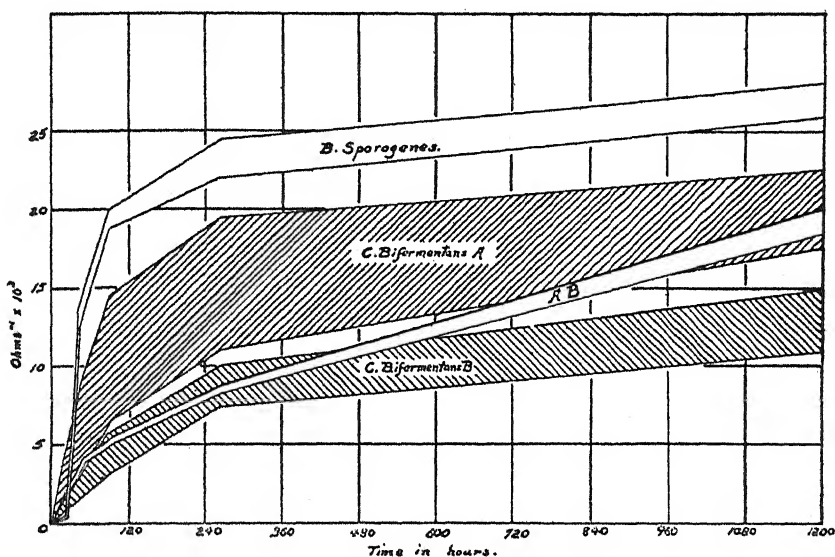
FIG. 2. RANGE OF CONDUCTIVITY CHANGE—OLD AGES

of their gelatin digestion is represented by the narrow zone, *AB*, which for the first forty-five hours lies within zone *A*, then deflects to enter zone *B* at about ninety hours, emerging again (see fig. 2) and reëntering zone *A*.   If, therefore, these two cultures should be included either in Group A or in Group B, the reconstructed zones would show a considerable overlapping and any such differentiation would be impossible.   Similarly, a grouping made according to the fermentation of sorbitol would give widely overlapping zones.

The difference between the bifermentans group and *C. sporogenes*, in gelatinolytic activity, is so clearly shown in the charts as scarcely to need comment. The latter shows a lag period extending for twenty hours, followed by a period of maximum activity, during which proteolysis, as measured by ammonia accumulation, proceeds to a point never attained by members of the bifermentans group.

TABLE 4

Grouping of bifermentans strains according to type of reaction

DIFFERENTIAL CHARACTERS	STRAINS, SOURCES AND PREVIOUS DESIGNATION			
	1 (Lister) "Bifermentans" 5 (Hall) "Bifermentans" 8 (Hall) "Centrosporogenes" 9 (Hall) "Centrosporogenes" 10–21 Authors' Strains	6 (Hall) "Centrosporogenes"	12 Authors' Strains 13 Authors' Strains	2 (Hall) "Bifermentans" 3 (Hall) "Bifermentans" 4 (Hall) "Bifermentans" 7 (Hall) "Centrosporogenes"
Rapidity of growth..........	A	A	A	B
Sensitiveness of motility....	A	A	A	B
Sporangium width...........	A	A	AB	B
Extent of hemolysis.........	A	A	A	B
Viscosity...................	A	A	B	B
Clear zone in gelatin........	A	A	B	B
Fermentation of sorbitol..	A	B	A	B
Rate of digestion........	A	A	AB	B

Consideration of these charts will readily show the possibilities of erroneous conclusions when observations of one or two strains are used as a basis for differentiation of anaerobic species. For instance, there are strains of *C. bifermentans* whose position lies nearer to the *C. sporogenes* zone than to the *B* zone.

Another important point illustrated by these charts is the possibility of fallacies arising from observations limited to one or two ages. Thus the conductivity readings for *C. sporogenes* indicate consecutively: first, coincidence with the less proteolytic strains of *C. bifermentans*; then, coincidence with the more pro-

teolytic strains; and finally, a distinct disparity from both.   The disagreement as to the extent of proteolysis caused by *C. bifermentans* may possibly be explained in this way.

Each of the characters studied indicates a greater difference between *C. sporogenes* and the bifermentans group than between the individual members of the group.   Correlation of the differentiations within the group indicates two types differing in morphology, hemolysis, viscosity, sorbitol fermentation and proteolysis.   The reaction common to strains 1, 5, 8, 9, 10, 11, and 14 to 21 inclusive are designated in table 4 as Type A reactions, and the reactions common to strains 2, 3, 4 and 7 as Type B reactions. Strain 6 has Type A reactions except on sorbitol.   Strains 12 and 13 have some Type A, some Type B, and some intermediate (AB) reactions.

While the reactions designated as Type A and Type B represent real differences, none of these differences, except sorbitol fermentation, are both sufficiently great and sufficiently constant to be recommended for general classification.   Furthermore, the fact that there are cultures giving, in some characters, Type A reactions, and in other characters, Type B or intermediate reactions, militates against using these reactions to divide the species.

Finally, it is to be noted that Hall's differential designation of his eight strains could not be substantiated by differences in any one of the characters studied.

<center>SUMMARY AND CONCLUSIONS</center>

With the assumption that *C. bifermentans* and *C. centrosporogenes* constituted a somewhat unified group, a study was made of all available strains (twenty-one) belonging to this group.

Since no consistent differences were found between the strains labelled *C. bifermentans* and the strains labelled *C. centrosporogenes*, the use of the latter name would seem unjustifiable.

The variations observed suggest another and a more logical grouping, but the nature of the variations and the existence of intermediate strains necessitate all of the strains being considered as a single species.

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USE OF THE MICROSCOPE IN STUDYING THE ACTIVITIES OF BACTERIA IN SOIL[1]

H. J. CONN

New York State Agricultural Experiment Station, Geneva, New York

The microscopic method for studying bacteria in soil was first proposed about twelve years ago (Conn, 1917). It was then employed chiefly as a method for checking up other procedures for counting bacteria in soil and as a means of determining the morphological types of microörganisms present in any given soil. It was felt at the time that its usefulness along these two lines was strictly limited, but it was believed that the future might show other ways in which it could be used to greater advantage. Such has proved to be the case. Winogradsky (1925) has employed the method (with slight changes in technic) for observing the kinds of organisms stimulated by the addition of various ingredients to soil. More recently Rossi (1927) has used a modification of the technic for studying the groupings and colony formation of bacteria in soil while Chudiakov (1926) has employed Winogradsky's technic for studying adsorption of bacteria by soil particles.

The line of investigation suggested by Winogradsky's work has been followed in this laboratory (Thatcher and Conn, 1927) along slightly different lines; and as a result of this study another use of the method has been worked out which promises to be fully as important as those in which it has been previously employed. The use in question is in studying the food requirements of bacteria in soil. The method has been used in this way in certain recent investigations (Conn, 1928a and 1928b) but it appears important enough for greater emphasis than that given in the publications cited.

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It has been recognized for some time that the behavior of soil organisms in soil and in laboratory media may be very different. Without question, it is more important to know their behavior in soil, but the difficulty of studying their physiology in such a medium has kept investigators from making much progress in this line.   In such a study three serious difficulties have always been encountered.   In the first place, pure culture work is impossible unless the soil is sterilized, and sterilization changes its physical and chemical nature.   In the second place, even if one assumes that the changes in the soil brought about by sterilization are not sufficient to affect results, one encounters great difficulty in making chemical analysis of the soil; while in the third place, the opacity of the medium renders it impossible to determine by eye whether growth has occurred or not.

The last two difficulties prove decided obstacles, because one cannot study the physiology of an organism unless one can measure its end products, or at least determine whether or not it is actually growing in the medium used for study.

In the method now proposed for the purpose, it must be stated frankly that the first of these difficulties has not been eliminated. No method has yet been found for studying the organisms in soil in pure culture without sterilizing the soil with heat.   It is believed, nevertheless, that even after autoclaving, soil is a more natural medium for soil bacteria than are either liquid or agar media.   In one or two instances it has been possible to check up the matter, in fact, by comparing the ability of an organism to grow in pure culture in a sterilized soil with its presence or absence on gelatin plates made from the soil in question; and the agreement has been close enough to suggest that heated and unheated soil are not so different as media for the growth of soil bacteria as was originally feared.

The real value of the microscopic method in the physiological study of soil bacteria is that it furnishes a rapid means of determining whether or not an organism is growing in a given soil sample.   In other words, it overcomes the handicap of working

with a medium in which the bacterial growth is invisible to the unaided eye.

In employing this method, a soil may be selected in which the organism under investigation does not grow unless special nutrients are added.   It may be possible to find a soil so poor in bacterial nutrients that no soil organism will grow in it without such addition.   As yet no effort has been made to locate such a soil, but it has proved simple to find one poorly adapted to the growth of the particular organisms thus far investigated.   Any nutrient material or mixture of nutrient materials that it is desired to study may be added to this soil; the soil may then be inoculated with the organism under investigation, and its ability to grow may be observed by microscopic examination.

TECHNIC EMPLOYED

The soil is nearly air-dried and passed through a sieve with holes about 2 mm. in diameter.   Water is added by spraying until the soil is moist, but still of a good consistency for handling.   It is then stored in stoppered bottles and when ready to use 10 grams are placed in each test tube.   The best sized test tube is one of 18 to 20 mm. outside diameter.   The tubes are plugged with cotton and sterilized thirty minutes at one atmosphere pressure in an autoclave.

The nutrients to be studied are generally added at the rate of 1 gram per 100 grams of soil, but the quantity employed may be varied according to the nature of the ingredient in question.   The ingredients may be added in one or the other of two different ways: they may be dissolved in the water sprayed onto the soil before sterilizing; or they may be dissolved in a portion of this water, sterilized separately by filtration and added aseptically to the soil after autoclaving.   The latter method is preferable in case of organic compounds subject to possible decomposition on heating.

The cultures used for inoculation are twenty-four-hour-old agar cultures; the growth is rubbed up in sterile distilled water and 1 cc. transferred under aseptic conditions to each tube of sterile soil.

After various periods, generally two days, four days, and six days, samples of 0.5 gram each are removed, with care to avoid contamination, and mixed with 4.5 cc. of a 0.015 per cent solution of gelatin. This gelatin solution is prepared previously and sterilized in test tubes. A drop of this suspension is smeared on a slide, and dried on a flat surface over a boiling water bath. It is then stained by the technic previously described by the writer for the study of bacteria in soil (Conn, 1918) and subsequently modified (Conn, 1928). This later modification is simpler and more controllable than the procedures previously suggested; it is as follows:

Make a suspension of soil in nine times its weight of an 0.015 per cent solution of gelatin. Smear a drop of this in a thin film on a slide. Dry on a flat surface over a boiling water bath. While still on the water bath cover with the following staining fluid:

Rose bengal... 1 gram
$CaCl_2$... 0.01 gram
5 per cent aqueous phenol............................... 100 cc.

Allow to stand for one minute. Wash as rapidly as possible in tap water; dry and examine under the microscope. A rather high power is necessary, the combination of lenses giving best results of any that have been tried being a 1.9 mm. fluorite objective with a 12.5 compensating or planoscopic ocular.

RESULTS OBTAINED

As an illustration of the use of this method two figures are given. Both of these figures show sterilized soil that was inoculated with an organism recently studied in this laboratory, *Bacterium globiforme*[2] Conn (1928a). Both illustrations are microscopic preparations of the same soil, Volusia silt loam, in which this organism does not ordinarily grow. In the case of figure 1 the soil had no treatment except sterilization. In figure 2 the soil contained 1 gram of ammonium sulfate and 1 gram of glucose per 100 grams of soil. These illustrations suggest that the reason this organism fails to grow in this soil under normal conditions is because the soil

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[2] Incorrectly spelled *Bacterium globiformis* when originally described.

lacks the necessary carbon or nitrogen sources, or both.   Other investigations already reported (1928a) show that this particular organism requires both carbon and nitrogen added to this soil in order to allow it to grow.   These two particular ingredients, however, (ammonium sulfate and glucose) are not the only ones satisfactory for the purpose.   The investigation has shown that this
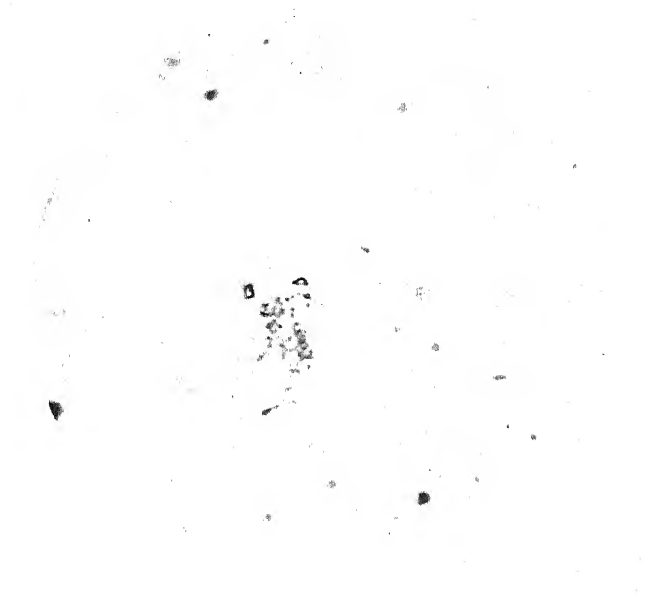
FIG. 1. SOIL THAT HAS BEEN LIMED, BUT WITHOUT OTHER ADDITION, STERILIZED, AND REINOCULATED WITH *Bact. globiforme*, ONE WEEK AFTER INOCULATION

Stained with rose bengal.   Magnification 1,000 diameters

organism has a wide choice among carbon and nitrogen sources and can employ carbon compounds as simple in composition as acetic acid.

The method is also being employed here on other soil bacteria and it is felt that it offers considerable promise.   It is now being called to the attention of others in the hope that it may be more generally employed.   Although it does not offer a means of study-

ing bacteria in unsterilized soil, nor of analyzing the end products of their growth in soil, it does furnish such a satisfactory method of determining their ability to grow in any soil, with or without the addition of nutrients, that it has distinct value in studying the activities of soil bacteria.

FIG. 2. SOIL THAT HAS BEEN LIMED AND TO WHICH HAS BEEN ADDED GLUCOSE AND AMMONIUM SULFATE, STERILIZED, AND REINOCULATED WITH *Bact. globiforme*, ONE WEEK AFTER INOCULATION

Stained with rose bengal. Magnification 1,000 diameters. Slightly retouched to improve the definition of the bacteria that were out of focus.

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THE INFLUENCE OF IRON ON THE PIGMENTATION OF ACID-FAST BACTERIA[1]

GUILFORD B. REED AND CHRISTINE E. RICE

Department of Bacteriology, Queen's University, Kingston, Ontario

Cultures of acid-fast bacteria have generally been observed to vary conspicuously in pigmentation. Colonies of tubercle bacilli growing on either liquid or solid media are generally white or nearly white in the initial stages of growth and usually become yellowish after a few weeks. Frequently the growing pellicle becomes deep yellow, occasionally brown or even reddish, while in other cases no pigmentation is observed at any stage of the growth. Much the same condition has been observed in the case of many of the saprophytic acid-fast organisms especially the smegma, grass and butter bacilli. The presence or absence of pigment in cultures of *M. leprae* has figured conspicuously in the controversial problem of the etiology of leprosy, Duval (1912).

We have used for some time in this laboratory, in the cultivation of both acid-fast and certain non-acid-fast organisms, a medium consisting of fresh infusion of 500 grams of beef, 10 grams peptone, 5 grams Na_2HPO_4, 7 grams glycerol and 15 grams of agar to a liter, autoclaved and adjusted to pH 7.4. For certain purposes this has been supplemented by the addition of 0.2 gram of ferric citrate per liter. Henley (1925) used a similar fluid medium. Analyses of the former, to which no iron was added, show that it contains less than 0.01 mgm. of iron per 100 cc.

All of the organisms mentioned in this paper grow at approximately the same rate on this medium, free of iron or with iron added, as judged by the mass of growth on the surface of agar

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plates or slants. In the case of the acid-fast species tested, there is, however, a great difference in the pigmentation of those grown on the iron-containing and the iron-free media. These results are summarized in table 1. Four cultures of *M. leprae*

TABLE 1

Pigmentation of certain acid-fast bacteria grown on organic media with and without iron

	12 DAYS GROWTH		30 DAYS GROWTH	
-	With iron	No iron	With iron	No iron
Human tubercle bacillus:				
Koch..........	No pigment	No pigment	Brown	No pigment
Upt. A.........	Creamy	No pigment	Yellow	No pigment
Irv............	No pigment	No pigment	Yellow	No pigment
S. N...........	No pigment	No pigment	Brown	No pigment
Bovine tubercle bacillus:				
599............	Deep yellow	No pigment	Deep yellow	No pigment
112..	Colorless	No pigment	Yellow	No pigment
B.C.G.........	Pale creamy	No pigment	Yellow	No pigment
Avian tubercle bacillus:				
10.............	Pale yellow	No pigment*	Yellow brown	Creamy
11.............	Pale yellow	No pigment	Yellow	No pigment
	4 DAYS GROWTH		10 DAYS GROWTH	
	With iron	No iron	With iron	No iron
M. leprae:				
Clegg I.........	Deep yellow	No pigment	Deep yellow	Creamy
Clegg II........	Deep brown	No pigment	Deep brown	Creamy
65 Duval.......	Brown	Pale yellow	Deep brown	Pale yellow
68.............	Brown	No pigment	Deep brown	Creamy
M. phlei.........	Brown	No pigment	Deep brown	No pigment
M. smegmatis....	Yellow	No pigment	Yellow	No pigment

were used, Clegg I and II obtained from the Lister Institute and numbers 65 Duval, and 68 from the American Type Culture Collection with cultures of *M. smegmatis* and *M. phlei* from the former collection. Cultures of these three species showed some

yellow pigment on the iron containing medium almost as soon as the colonies could be distinguished and at the end of four days growth they were deeply pigmented, while at the same time a similar amount of growth on the iron-free medium remained without pigmentation.

The very rapidly growing tubercle bacillus, bovine 599, obtained from the American Type Collection produced a deeply pigmented growth on the surface of the iron-containing medium in six to eight days, while a similar growth on the iron free medium remained non-pigmented. A culture of Calmette's B.C.G. produced a pale yellow growth in ten to twelve days and a deep yellow pigmented growth in twenty to thirty days on the medium with iron while cultures on the iron-free medium exhibited no color. Two recently isolated avian strains were both slightly pigmented on the iron-containing medium after ten days and deeply pigmented after thirty days growth, in contrast with the white or at most cream colored growth on the iron-free medium. Several slower growing cultures of the tubercle bacillus, human Koch strain, from the Lister Institute, three cultures recently isolated from sputum of pulmonary cases and bovine 112 from the American Type collection formed definite colonies by the tenth to twelfth day though they were without color on both media but by the thirtieth day the growth on the iron-containing agar was conspicuously pigmented while similar sized colonies on the iron-free medium remained colorless or white.

In sharp contrast to the behavior of the acid-fast forms all the pigmented non-acid-fast bacteria tested, including several species of the genera, Staphylococcus, Flavobacterium, Pseudomonas and Bacillus produced approximately equal amounts of pigment on both the iron-free and the iron-containing media, table 2.

When the tubercle bacilli of this series were grown on the synthetic medium, described in a previous paper (Reed and Rice, 1928), consisting of asparagin, Na_2HPO_4, NaCl, Na citrate, and ferric sulphate a heavy deep yellow pellicle developed in two to five weeks. On the same medium except for the absence of iron, a similar mass of pellicle, although as previously shown less in weight, exhibited no pigmentation.

The presence of citrate in this medium, as previously shown (Reed and Rice, 1928), stabilises the iron, probably through the formation of complex ions, so that the iron remains in solution up to approximately pH 8.0. In more alkaline solutions, even in the presence of the citrate, iron is precipitated. Corresponding with this, the 599 bovine tubercle bacillus growing on this medium at more acid reactions than pH 8.0 shows definite pigmentation while a more alkaline reaction of the medium results in growth

TABLE 2

Pigmentation of certain non-acid-fast bacteria grown on media with and without iron

	WITH IRON	WITHOUT IRON
Staphylococcus:		
Species 1	Deep yellow	Pale yellow
Species 2	Creamy	Creamy
Species 3	Lemon yellow	Lemon yellow
Species 4	White	White
Species 5	Pink	Pink
Flavobacterium:		
Species 1	Brown	Yellow
Species 2	Yellow	Yellow
Pseudomonas:		
Flourescens	No pigment	Green pigment
Species	Green pigment	Green pigment
Bacillus:		
Species 1	Pale yellow	Pale yellow
Species 2	Pink	Pink

without pigment. This possibly accounts for the fact that Long and Sibert (1926) found only slight increase in growth in their synthetic medium on addition of iron and also that the pellicle was pure white.

CONCLUSIONS

Data have been presented which indicate that the yellow, brown or red pigmentation of acid-fast bacteria is related to the presence of iron in the culture media. Lack of stabilizing substances or high pH which permits precipitation of the iron pre-

vents pigment formation. Non-acid-fast bacteria are not so affected by the iron content of culture media.

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CLASSIFICATION OF THE STREPTOCOCCI OF HUMAN FECES[1]

HENRY WELCH

From the Department of Bacteriology, Brown University, Providence, Rhode Island

The use of fermentative reactions in carbohydrate media for tracing the relationships of the streptococci was first suggested by Gordon in 1904 and elaborated by Houston in 1905 and by Andrewes and Horder in 1908. The last two were able, from their own results and those of Gordon, to divide the streptococci into seven sub-groups or species which they considered main types or type centers about which the more variable forms were clustered. This paper is concerned with their sixth type, *Strep. fecalis,* which ferments sucrose, lactose, salicin, coniferin and mannitol.

In general, the problem resolved itself into the isolation and differentiation of the streptococci found in some thirty specimens of human feces. Over five hundred cultures were isolated and classified according to their fermentative reactions.

The media used were prepared according to standard methods. It was found, however, that the best growth was obtainable at a pH of 7.2 to 7.4 and, furthermore, that when 1 per cent each of glucose and lactose was added to the agar plates a more vigorous growth was produced.

In isolating the streptococci, a portion of a fresh stool was mixed thoroughly with sterile saline solution and one loopful smeared over three agar plates. After a forty-eight-hour growth the colonies of streptococci were located with a hand lens. In order to insure growth it was found necessary to cut the agar bordering the colony and to transfer the whole colony to a glucose sugar broth tube. Although the chance of contamination was

[1] This research was carried out under the direction of Dr. C. A. Stuart, Professor of Bacteriology, Brown University, Providence, R. I.

increased, it was found that by using the hand lens any minute colonies in close proximity to the colony chosen could be detected and avoided. The formation of acid without gas in forty-eight hours was considered a partially confirmed test for streptococci and the test was considered complete with microscopic demonstration of the morphology.

Morphologically, all the strains found were much larger in diameter than the true hemolytic streptococci (i.e., the Beta and Gamma types of the Brown Classification). There are fewer cocci to a chain; the cocci grow much better on agar than the true hemolytic streptococci, but by no means can their growth on agar be called prolific. Only in one strain (VI) was the growth on agar good. In all the other five strains the growth on agar was scanty and veil-like, sometimes difficult to see unless held in direct light. The morphology of the streptococci does not help much in their differentiation. When grown on different media few changes take place. It was found that the chains could be lengthened somewhat by the use of broth to which a small amount of blood serum had been added. It was also found that the diameter of the cocci could be increased by growth on a specially prepared peptic digest medium. On the whole, the morphology was as constant as the fermentative reactions, which did not vary during the time they were under investigation.

PHYSIOLOGY

The streptococci isolated were first differentiated by their fermentative reactions. During the earlier part of the research twelve carbohydrates were used: glucose, lactose, sucrose, salicin, maltose, mannitol, dulcitol, inulin, dextrin, xylose, raffinose and galactose. After two months' work with these twelve carbohydrates it seemed advisable, because of their continued negative results, to drop inulin, xylose, dextrin, raffinose and dulcitol. At no time were the streptococci able to form acid in these five carbohydrates.

During the investigation cultures of the various strains were from time to time saved as stock. Some of these cultures were carried eight months and some more than fifteen months. Every

two weeks the stock cultures were checked in the seven sugars used in order to determine whether they still gave the same fermentation reactions; but in no case did they vary.

Six distinct types were isolated and differentiated according to their fermentative ability. All types do not necessarily appear in a single stool although in the majority of cases such was the case. The types isolated gave the reactions shown in table 1.

Strain I is the *Strep. fecalis* which has been isolated and classified previously. The other five are types that vary definitely from the type of Andrewes and Horder.

A culture of each type has been studied for over fifteen months and each gives the same fermentative reactions that it did when isolated.

TABLE 1*

STRAIN NUMBER	GLUCOSE	LACTOSE	SUCROSE	SALICIN	MALTOSE	GALACTOSE	MANNITOL
I	+	+	+	+	+	+	+
II	+	+	−	+	+	+	+
III	+	+	−	+	+	+	−
V	+	+	+	+	+	+	−
VI	+	+	+	−	+	+	−
VII	+	−	+	+	+	+	+

* In January, 1929, two years after beginning the research, I had the opportunity to recheck the fermentative reactions of the cultures as listed in this table. The reactions were unchanged.

Specimens from cases of jaundice, dysentery and typhoid were used, as well as normal stools, and it is an interesting fact that the various strains appeared in about the same percentage in the abnormal as in the normal stools.

It seemed advisable to determine whether or not any of the strains isolated would produce hemolysis of red cells. Each was accordingly inoculated in blood plates and grown for forty-eight hours. Strains I, III and V produced a small clear zone of hemolysis about the colonies and there was a slight greenish tinge on each of the three plates. This type of reaction is similar to that produced by *Strep. viridans*, and according to the Brown Classification these strains would be classified under the Alpha

type. However, the hemolysis found was not by any means similar to that shown by the true hemolytic streptococcus, being much smaller in area. Strains II, VI and VII produced no hemolysis and no methemoglobin. In order to determine the serological relationship of the strains isolated, six vaccines were made, one from each strain. Over a period of eight weeks increasing amounts of the vaccines were injected into rabbits in five-day intervals. At the end of the period the following titers were obtained:

Strain   I, dilution 1–40960
Strain   II, dilution 1–160
Strain III, dilution 1–10240
Strain   V, dilution 1–40960
Strain VI, dilution 1–160
Strain VII, dilution 1–80

TABLE 2

STRAIN NUMBER	SERA I	SERA III	SERA V	SERA II	SERA VI	SERA VII
I	40960	5120	10240*	10240	640	640
III	10240	10240	5120	10240	1280	640
V	10240	5120	20480	10240	1280	640
II	80	40	20	160	20	40
VII	10	80	10	80	10	40
VI	Negative	Negative	Negative	Negative	160	Negative

* *Strep. fecalis.*

The serological relationship was determined by cross agglutination tests the results of which are given in table 2.

Figure 1 which shows this serological relationship graphically, shows at a glance the rather close relationship of strains I, III and V in comparison to strains II, VI and VII. The figure is plotted on a semi-logarithmic basis in order to include all strains— some having very low dilutions, while others had comparatively high ones.

The cross agglutination tests would indicate a grouping of strains I, III and V and of strains II and VII, whereas strain VI is apparently in a class by itself.

Strains I, III and V, it will be seen, are strong both serologically and antigenically; strains II and VII are weak serologically and fair antigenically, whereas strain VI is negative serologically and only fair antigenically.
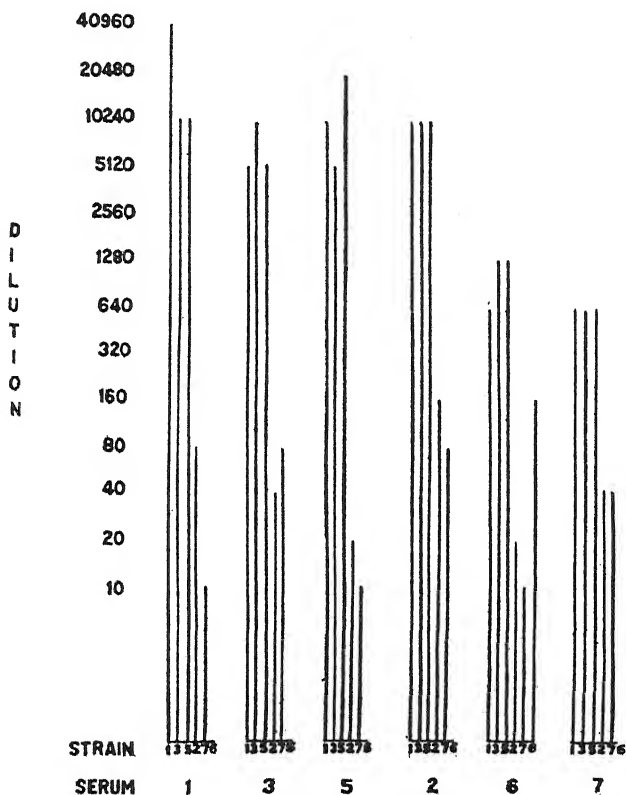
FIG. 1. SEROLOGICAL RELATIONSHIP OF STRAINS 1, 3 AND 5 COMPARED WITH STRAINS 2, 6 AND 7 (SEMI-LOGARITHMIC)

DISCUSSION

In a study of streptococci, not only fecal forms but others, it is extremely difficult to differentiate strains by fermentative reactions. Most workers on the subject have found this true because of the variation in fermentation. Gordon and Houston in 1904, Andrewes and Horder in 1906, and Winslow and Palmer

in 1910, all claim a variation in the strains studied. Many therefore have differentiated the streptococci by the percentage of acid produced in the sugars, glucose being the most popular. Winslow and Palmer concluded from their investigations that the presence of over 3.5 per cent acid in glucose was characteristic of human stools. Secondly, that raffinose-fermenting forms appear to be more abundant in bovine than in human feces, and thirdly, that mannitol-fermenting forms make up about one quarter of human streptococci and are very rare in the feces of the horse and cow.

From the point of view of this paper all the results obtained by these last mentioned investigators coincide closely with the results of this study. In the first place no streptococci were isolated which were not able to produce some acid in glucose. Secondly, no raffinose-fermenting forms were isolated and, thirdly, about 25 per cent of the forms isolated fermented mannitol.

The question arises as to whether such fermentative reactions as were made are fair tests for differentiation. Do the streptococci maintain their fementative characteristics over long periods of time? This investigation would indicate that such is the case. The six strains were carried on agar at a pH of 7.2 for over fifteen months and they still ran true to form, giving the identical fermentative reactions that were obtained on direct isolation from fresh stools.

Philip Hadley of Michigan notes (personal communication) that he and his co-workers have found a variation in *Strep. fecalis* in morphology and in fermentative characters caused by a variety of poor environments.

No work was done in this investigation along these lines. The streptococci were carried on standard agar media. Probably variation can be forced, but if one maintains fixed fermentative characters by the use of standard media, it seems possible to differentiate the strains (at least of the feces) in this way.

P. G. Heineman (1915) states that "The fermentative ability of his two strains was not changed materially by repeated transfers through litmus milk." He further states that these fermentative reactions are of particular interest. Several earlier

papers by the same author deal with the variability in fermentative reactions of several other species of bacteria.

Goodman (1908) succeeded in producing a high acid and a low acid form of diphtheria bacilli from one strain. Winslow and Walker (1909) found acid formation by *B. paratyphosus* relatively stable, but made no attempt at variation of environment. Buchanan and Truax (1910) tested inheritance of acid production of *Strep. lacticus*. They concluded that "impressed variations do not appear to be inheritable." Rettger and Sherrick (1911) conclude from a study of several organisms that bacterial variation, at least of fluctuating type, may be brought about by what may be termed "artificial selection."

E. C. Rosenow (1914) in his paper on streptococcus-pneumococcus transmutations, states that the amount of hemolysis on blood plates was not lost but only decreased by repeated transference on such plates.

These conflicting results indicate that in the field of "Microbic Dissociation" (Hadley, 1927), there is much that awaits further experimentation.

There is little doubt that practically all forms of bacteria can be changed in morphological and fermentative characteristics by the use of a variety of environments including passage through animals, but even so this does not clear up the situation, and even involves it more deeply.

If we are to take Hadley's theory of "Microbic Dissociation" as a criterion, then there is no classification of streptococci, fecal or otherwise. Either we must consider fixed and definite fermentative, morphological, and physiological characteristics, or cast aside the previous classifications and consider only the variety of characteristics obtainable through enforced variation.

The streptococci of the feces as studied in this investigation, without the use of unfavorable environments, were constant in their physiological and morphological characters. It would seem, then, that a classification based on the fermentative ability of the streptococci would be logical.

The *fermentative results* indicate that besides *Strep. fecalis* there are five other species of the streptococci of the feces that differ in their fermentative ability.

According to *serological results* the streptococci of the feces are divided differently. There are apparently three groups. These are considered in this paper as follows: group A strains I, III and V; group B, strains II and VII, and group C, strain VI. The strains of group A are strong both antigenically and serologically, those of group B are weak serologically and fair antigenically, while strain VI is negative serologically and only fair antigenically.

On the basis of blood plates the streptococci are divided into two groups. Strains I, III and V all gave a slight hemolysis, which was not true of strains II, VI and VII.

From these two sections of the investigation, i.e., the physiological and serological, the indication is that there are at least two and possibly three separate and distinct types of the streptococci of the feces.

The strains I, III and V (strain I being *Strep. fecalis*) are all closely related; they show no apparent difference in morphology, close relationship on blood plates and close relationship, serologically, although having varying fermentative reactions.

Strains II and VII are also closely related in morphological and serological characteristics.

Strain VI, however, differs considerably from all other strains. It has a growth on agar that makes it easily recognized among all the rest. It is negative serologically and the general results of the work indicate that strain VI is quite separate from the other five strains.

The primary idea of the investigation was to determine whether or not there were in the human feces other streptococci besides *Strep. fecalis*. The results indicate that such is the case. Whether these forms may be called distinct species is an open question. Certainly strain VI, with its negative serological characteristics and peculiar morphology, is distinct from the other five. But whether there are enough distinctive characteristics of the strains I, II, III, V and VII to separate them into species is more doubtful and needs further work.

SUMMARY

The streptococci of the feces are apparently constant in their fermentative ability.

Differentiations should be made only in media which are most favorable for the growth of the organism.

The results indicate that there are six strains common to human stools. Those fermenting (a) all sugars used, glucose, lactose, sucrose, salicin, maltose, mannitol, galactose; (b) all but sucrose; (c) all but sucrose and mannitol; (d) all but mannitol; (e) all but mannitol and salicin; (f) all but lactose.

Serologically, strains (a), (c) and (d) resemble each other and give slight hemolysis. Strains (b), (e) and (f) give no hemolysis and of these (b) and (f) resemble each other serologically while (e) is distinctly different.

There is little in the morphology of the streptococci of the human feces that assists in their differentiation.

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THE GERMICIDAL ACTION OF HALOGEN DERIVATIVES OF PHENOL AND RESORCINOL AND ITS IMPAIRMENT BY ORGANIC MATTER[1]

EMIL KLARMANN, VLADIMIR A. SHTERNOV AND JOHN VON WOWERN

*Plaut Research Laboratory, Lehn and Fink Products Company,
Bloomfield, New Jersey*

INTRODUCTION

A considerable amount of research work has been published in the chemical, biological and medical literature from which a number of conclusions have been drawn regarding the relationship between chemical constitution and disinfectant action. There are also many papers on record which contain a more or less successful attempt to visualize the mechanism of disinfection and to define it, if possible, chemically or physically. Although the disinfectant action of phenol and its derivatives has been known for some time, the ultimate mode of its action upon the microörganisms remains unknown. An attempt has been made by Loew (1893) to correlate the germicidal action of phenol with its reactivity toward aldehyde groups of the protoplasm. Cooper and collaborators (Cooper, 1913; Cooper and Sanders, 1927; Cooper and Mason, 1928) attribute the disinfectant action of phenol to its capacity of acting as a protein precipitant. The assumption made by Loew cannot hold in all cases because it is known that certain phenol derivatives, such as pentahalogen phenol, are very potent germicides under certain conditions, although by virtue of their constitution they cannot be regarded as capable of a condensation reaction with aldehyde groups. Regarding the work by Cooper and his collaborators, one may

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say that the precipitation of proteins by phenol and its derivatives may very well be preceded by a chemical reaction between the disinfectant chemical and the protein; besides, it appears from a number of references in the literature, that disinfectant action in the presence of protein need not be accompanied by the precipitation of the latter.

From the findings of other investigators, hitherto published, it is rather difficult to obtain a definite idea which could be made the basis of a working hypothesis for the subject under consideration. With the experimental evidence on hand it appears to be impossible to say whether a simple penetration of the disinfectant compound into the living cell or a change of the physical-chemical conditions within, or a chemical reaction with definite cell constituents, or some other process, is responsible for what eventually appears to be the "germicidal action." More recently there has been a tendency to attribute the germ killing action to more or less definite chemical reactions, (e.g. formation of salts or salt like compounds), which might be accompanied by other reactions (Schneider, 1926).

An attempt to base the bacteriostatic action of organic dyes upon the assumption of a reaction between definite chemical groups was made by Stearn and Stearn (1926). According to Simon and Wood (1914) there are in the bacterial organism receptors for either acidic or basic substances; this might be represented by a type formula $R\dfrac{NH_2}{COOH}$ the amino group acting as a receptor for acid substances and the carboxyl group as a receptor for basic ones. A simpler formula would be HPrOH. Simon and Wood assume that the dye enters into a peptide like combination with the cell-protein which according to Stearn and Stearn might in the case of a basic dye be represented by the equilibrium

$$D^+ + PrOH^- \rightleftharpoons DPrOH$$
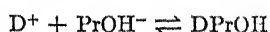
where D^+ is the cation of the dye DOH, $PrOH^-$ the anion of the receptor HPrOH and DPrOH the un-ionized dye-protein compound.

The participation of a definite chemical group in the bacteri-

cidal action of organic arsenicals was assumed by Voegtlin, Dyer and Leonard (1925).   These authors believe that the effectiveness of arsenicals depends upon their reaction with the SH-group; the latter occurs in compounds such as cystein and glutathione, which, as is known from other work, are essential for a number of biological processes taking place in the cell.   It is, therefore, clear that the inactivation of such compounds, will lead to serious interference with the life process of the cell and ultimately to its death.   The conclusions of Voegtlin and collaborators have been disputed by Schnitzer (1928).

The determination of specific chemical groups of cell substances which play a part in the fixation of the toxic molecule, has not as yet been accomplished directly, but such determination might be attempted by a process of elimination.   Thus, if it be found that certain substances inhibit the germicidal action, e.g., of phenol, one could assume that groups which are responsible for the fixation of phenol to this substance and its inactivation in a germicidal sense, are also present in the cell.   It is our intention to use this procedure in our attempts to contribute to the elucidation of the mechanism of disinfection by phenol, resorcinol and their halogen derivatives.

From older work, which has direct reference to the germicidal action of polyhalogen phenol derivatives and its reduction by organic matter, the paper by Bechhold and Ehrlich (1906) appears to be the most important.   These authors endeavored to find a compound which would be satisfactory for "internal" antisepsis, i.e., would show a pronounced bacteriotropic and at the same time a minimal organotropic action.   In the series of the halogen derivatives of phenol, they tested tri-, tetra- and pentahalogen phenol.   They conclude from their work that "the introduction of halogen (Cl, Br) into phenol increases its germicidal action with the number of halogen atoms introduced (1 molecule of penta-bromo-phenol having the same effect upon *B. diphtheriae* as 500 molecules of phenol)" and that "the most effective disinfectants (such as tetra-bromo-o-cresol or tetra-chloro-o-biphenol) fail to act in the presence of serum, although they do not precipitate it."   These conclusions would lead one to assume that

halogen substitution produces compounds, the germicidal efficacy of which is impaired by organic matter.   It was later shown, however, by Laubenheimer (1909) that mono-halogen substituted alkyl derivatives of phenol, although much more powerful than the unsubstituted compounds, are only partly inactivated in the presence of serum.

An important contribution to the problem of inactivation of phenol derivatives by organic matter has been made by Sabalitschka (1928).   This author has shown that while the germicidal effect of p-hydroxy-benzoic acid is very considerably reduced in the presence of protein matter, the efficacy of its alkyl esters is practically the same as in the absence of organic matter.   He explains this by assuming that the compound with a free carboxyl group, is capable of entering into chemical combination with the organic matter, which does not take place in the case of the ester.

Attention should finally be called to a paper by Klarmann and Von Wowern (1929), who studied the influence of halogenation of 2,4-dihydroxydiphenylmethane upon the germicidal efficacy and its reduction by organic matter.   In this case, also, a very considerable increase of germicidal efficacy was observed, different results having been obtained depending on whether the halogen was introduced into the dihydroxy-substituted or the unsubstituted nucleus.   The reduction of germicidal efficacy of these compounds by standard[2] organic matter was very pronounced.

EXPERIMENTAL

We assumed that a closer investigation of the disinfectant action of the halogen derivatives of phenol and resorcinol by means of standard methods and of its impairment by organic matter, would simplify the classification of the various subproblems and would lead to a choice of satisfactory chemical and bacteriological testing material for further work.

Standard bacteriological technique

In this investigation, two microörganisms, namely *B. typhosus* and *Staph. pyogenes-aureus*, were used.   Fresh twenty-four-hour

[2] As specified by the method of the Hygienic Laboratory (1912).

broth cultures were taken.   The composition of broth was as follows: Liebig's beef extract "Lemco" 0.5 per cent, "Difco" peptone 1 per cent and sodium chloride 0.5 per cent, reaction adjusted to pH 6.8.   The germicidal efficacy of the various compounds was tested by exposing 0.5 cc. of culture to the action of 5 cc. of the diluted disinfectant for a period of five, ten and fifteen minutes respectively, transferring a loopful into fresh media and incubating for forty-eight hours, after which the final readings were made.   In experiments with organic matter, a standard mixture was used consisting of 10 per cent "Difco" peptone and 5 per cent gelatin, 1 cc. of this mixture being added to 5 cc. of

TABLE 1

Phenol; minimum concentrations required for killing B. typhosus and Staph. aureus

	B. TYPHOSUS									STAPH. AUREUS								
	A without organic matter				B with organic matter					A without organic matter				B with organic matter				
	Time of exposure									Time of exposure								
	5 minutes	10 minutes	15 minutes	Average	5 minutes	10 minutes	15 minutes	Average	Ratio $\frac{B}{A}$	5 minutes	10 minutes	15 minutes	Average	5 minutes	10 minutes	15 minutes	Average	Ratio $\frac{B}{A}$
Dilutions (average of 25 experiments):																		
Calculated....	81.6	90.4	96.0	89.3	69.6	75.6	80.4	75.2	0.84	54.4	58.8	61.2	58.1	42.0	50.0	50.0	47.3	0.81
Practical......	80	90	100	90.0	70	75	80	75.0		55	60	60	58.3	40	50	50	46.6	

the disinfectant prior to the addition of 0.5 cc. of culture, so that in the actual test 2 per cent of peptone and 1 per cent of gelatin were present.

RESULTS

The resistance of the germs used may be seen from table 1, containing the maximum dilutions of phenol at which *B. typhosus* and *Staph. pyogenes-aureus* are killed, both in the absence and presence of organic matter at 20° after five, ten and fifteen minutes.   The average figures have been calculated from 25 generally agreeing determinations.

It appears from this table that the presence of organic matter effects a reduction of the germicidal efficacy of phenol against both *B. typhosus* and *Staph. pyogenes-aureus*; the reduction is not very pronounced, the average ratio of the germ killing power in the absence and the presence of organic matter being 0.84 in the case of *B. typhosus* and 0.81 in that of *Staph. pyogenes-aureus*.

In order to ascertain the influence of halogen substitution upon the bactericidal efficacy and its reduction by organic matter, a

TABLE 2

Halogen derivatives of phenol; phenol coefficients

	B. TYPHOSUS				STAPH. AUREUS			
	Without organic matter	With organic matter			Without organic matter	With organic matter		
		Calcu-lated	Cor-rected	Reduc-tion of efficacy		Calcu-lated	Cor-rected	Reduc-tion of efficacy
				per cent				*per cent*
o-Chloro-phenol............	3.6	3.7	3.1	14	3.8	3.7	3.0	21
p-Chloro-phenol...........	3.9	3.8	3.2	16	4.0	4.1	3.3	17
m-Chloro-phenol..........	7.4	6.2	5.2	30	5.8	5.2	4.2	27
2,4-dichloro-phenol........	13.3	11.4	9.6	28	12.7	12.2	9.9	22
2,4,6-trichloro-phenol.....	22.6	15.2	12.8	44	25.0	12.9	10.4	58
2,4,6-trichloro-phenol saturated aqueous solution....	21.1							
o-Bromo-phenol...........	3.8	3.4	2.8	25	3.7	3.1	2.5	32
p-Bromo-phenol...........	5.4	4.8	4.0	25	4.6	5.0	4.1	12
2,4-dibromo-phenol.	18.8	18.0	15.1	20	21.9	17.7	14.3	35
Phenol....................	1	1	0.84	16	1	1	0.81	19

series of halogen derivatives of phenol was tested, the results of which appear in table 2.[3]

[3] We have found that initial solutions are prepared conveniently by dissolving the chemical in alcohol first and then diluting with water. The final solutions did not, however, contain more than 5 per cent alcohol with exception of the case of tri-chloro-phenol. A small quantity of alcohol does not influence the results to any great extent; of course, appreciable quantities of alcohol may be expected to change the findings markedly since alcohol is known to influence the bactericidal properties of organic compounds in both ways (Koch, 1881).

In the case of tri-chloro-phenol, a larger quantity of alcohol had to be applied because of the unsatisfactory solubility of tri-chloro-phenol in water. Thus

According to this table, the germicidal efficacy increases directly with the number of atoms of halogen introduced. Bromo-substitution leads to compounds which are germicidally more active than the corresponding chloro derivatives. The degree of reduction of bactericidal efficacy by organic matter also depends upon the number of halogen atoms in the compound. While in the columns under the heading "With Organic Matter Calculated" the germicidal efficacy of phenol is regarded as

<div align="center">TABLE 3</div>

Certain alkyl phenols and their halogen derivatives; phenol coefficients

	B. TYPHOSUS				STAPH. AUREUS			
	Without organic matter	With organic matter			Without organic matter	With organic matter		
		Calculated	Corrected	Reduction of efficacy		Calculated	Corrected	Reduction of efficacy
				per cent				*per cent*
m-Cresol...................	2.5	2.4	2.0	20	2.4	2.4	1.9	19
4-chloro-m-cresol.........	30.5	26.1	21.9	28	19.5	12.2	9.9	49
Xylenol...................	5.0	5.0	4.4	12	5.6	4.0	3.2	42
Chloro-xylenol............	70.9	60.3	50.6	29	38.7	11.1	9.0	77
Thymol...................	33.2	32.5	27.3	18 ·	23.6	11.5	9.3	61
4-chloro-thymol...........	61.3	25.9	21.7	65	158.0	70.7	57.3	64
4-Bromo-thymol...........	12.5				194.5			
Carvacrol.................	39.9	30.6	25.7	36	17.8	6.0	4.7	73
4-bromo-carvacrol.........	25.0				126.7			
Phenol...................	1	1	0.84	16	1	1	0.81	19

unity, under the column with the heading "Corrected," the figures are based upon the germicidal efficacy of phenol in the absence of

the final dilution in which no growth of *B. typhosus* occurs, viz., 1:2000, contains 9 per cent of alcohol; that in which no growth of *Staph. pyogenes-aureus* occurs, viz., 1:1500, contains 11 per cent of alcohol. Therefore, the figures in the fifth horizontal column of table 2 do not represent entirely objective values; on the other hand it is not probable that the alcohol plays an important rôle even in this case, because a saturated solution of tri-chloro-phenol in water, which corresponds very nearly to a dilution of 1:2000, gives a phenol coefficient of 21.1 (with horizontal column) based upon the dry content, in satisfactory agreement with the figure of 22.6 obtained with a solution containing 9 per cent alcohol.

organic matter being 1.   m-Chloro-phenol seems to be an exception, in that it is germicidally more active, and the reduction of its germicidal efficacy by organic matter is more pronounced, than in the case of its isomers.

There is also a direct relationship between the degree of reduction of the germicidal power by organic matter and the number of halogen atoms in the phenol derivative.

Table 3 illustrates the influence of halogenation of certain alkyl derivatives of phenol upon the germicidal action.   In all cases halogenation produces a very marked increase of germicidal efficacy.   Considerable impairment of the disinfectant action is observed in the case of certain alkyl derivatives of phenol, the difference between the efficacy in the absence and in the presence of organic matter being particularly noticeable in tests with *Staph. pyogenes-aureus*.   Thus m-cresol and xylenol show about the same germicidal efficacy against *B. typhosus* and *Staph. pyogenes-aureus* and there is no pronounced impairment of the germicidal efficacy by organic matter.   On the other hand, organic matter reduces the disinfectant action of thymol and carvacrol against *Staph. pyogenes-aureus* more than 50 per cent, while the reduction in the case of *B. typhosus* is comparatively insignificant.

The germicidal efficacy of 4-chloro-m-cresol is considerably greater than that of the unsubstituted m-cresol, the increase being much more pronounced against *B. typhosus* than *Staph. pyogenes-aureus*.   The impairment of germicidal action by organic matter is somewhat more pronounced in the case of *Staph. pyogenes-aureus*.   Still higher figures are obtained with chloroxylenol, the germicidal efficacy of which is very markedly reduced by organic matter in the case of *Staph. pyogenes-aureus*.   With chlorothymol one has the strange phenomenon of the germicidal efficacy against *Staph. pyogenes-aureus* being much more pronounced than against *B. typhosus*.   With bromothymol and bromocarvacrol similarly high figures against *Staph. pyogenes-aureus* are obtained in the absence or organic matter.   However, extremely varying results appear in other cases; it is impossible

to account for these variations at the present time (they were, therefore, left out in the table).[4]

The series of halogen derivatives of resorcinol was investigated in a similar manner.   Although the introduction of alkyl groups into the nucleus of phenol is known to lead to derivatives showing great germicidal potency [Simon and Wood (1914)], one finds that halogenation of resorcinol, although leading to compounds which are much stronger disinfectants than resorcinol itself, does not produce derivatives of unusually high germicidal action, comparable, e.g., to that of the alkyl derivatives of resorcinol; this is clearly in contrast to the corresponding conditions in the case of phenol.

TABLE 4

Resorcinol and its chloro and bromo derivatives; phenol coefficients

	B. TYPHOSUS				STAPH. AUREUS			
	Without organic matter	With organic matter			Without organic matter	With organic matter		
		Calculated	Corrected	Reduction of efficacy		Calculated	Corrected	Reduction of efficacy
				per cent				*per cent*
Resorcinol...............	0.3	0.3	0.25	17	0.4	0.3	0.24	39
4-chloro-resorcinol........	0.7	0.5	0.42	40	1.0	0.6	0.48	52
4,6-dichloro-resorcinol....	3.2	2.0	1.7	47	3.9	2.2	1.8	54
2,4,6-trichloro-resorcinol..	5.0	2.3	1.9	61	4.3	2.4	1.9	55
4-bromo-resorcinol........	1.0	1.0	0.84	16	1.25	0.8	0.65	48
4-6-dibromo-resorcinol....	4.0	2.7	2.3	43	4.5	2.0	1.6	64
2,4,6-tribromo-resorcinol..	6.7	2.9	2.4	64	6.4	2.7	2.2	66

Table 4 illustrates this.   Mono substitution of resorcinol leads to compounds which are 2 to 3 times more germicidal than resorcinol but not stronger than phenol.   The di-substituted compounds are 11 to 13 times, and the tri-substituted 16 to 22 times more germicidal than resorcinol.   As in the case of phenol, bromo

[4] Most compounds enumerated in table 3, and particularly the halogen derivatives, are extremely little soluble in water.   They were, therefore, used in soap emulsions for the bacteriological test.   In view of the well known fact that the quantitative relationship between the amount of soap and that of the disinfectant compound sometimes influences the germicidal efficacy of the latter, the figures in table 3 are of relative importance.

substitution leads to compounds which are more germicidal than those obtained by substitution with chlorine. Organic matter effects a reduction of the germicidal efficacy on the average of more than 50 per cent in the case of the di- and tri-substituted halogen derivatives of resorcinol.

Influence of the media upon the bactericidal action

The considerable reduction of disinfectant action by organic matter previously dealt with suggested the possibility that the small quantity of organic matter present in the media might affect the disinfectant action to some extent. In other words, it was reasonable to suppose that if the culture were entirely freed from its media, it would be more accessible to the action of the disinfectant than when brought in contact with the disinfectant solution with the media adhering. In order to determine whether this is the case, tri-chloro-phenol was tested first, because in the case of this substance, the most distinct reduction of germicidal efficacy by organic matter was expected. When the experiment corroborated our surmise with reference to this chemical, the investigation was extended to dichlororesorcinol. This substance was chosen because it combines the advantage of satisfactory solubility in water with a sufficient sensitiveness of germicidal efficacy to the presence of organic matter. It was also necessary to make sure that the viability of the germ was not markedly impaired by the process of centrifuging. This was done by testing the resistance of the germ against phenol under the same conditions.

The following procedure was adopted: 10 cc. of culture was first centrifuged and the clear supernatant liquid drawn off. Then the bacterial residue was stirred up with a sterile platinum wire and 10 cc. of sterile physiological salt solution added. After thorough mixing the tube was centrifuged. The supernatant liquid was drawn off and the residue stirred with a fresh portion of physiological salt solution and filtered. Five-tenths cubic centimeter of the suspension was removed and mixed with the diluted disinfectant. It may be assumed that by this method practically all organic matter is removed.

TABLE 5

Phenol, trichloro-phenol, dichloro-resorcinol; a comparison of the dilutions required for killing "washed" and "not washed" B. typhosus and Staph. aureus

	B. TYPHOSUS										STAPH. AUREUS									
	Without organic matter					With organic matter					Without organic matter					With organic matter				
	5 min-utes	10 min-utes	15 min-utes	Average	Ratio B/A	5 min-utes	10 min-utes	15 min-utes	Average	Ratio B/A	5 min-utes	10 min-utes	15 min-utes	Average	Ratio B/A	5 min-utes	10 min-utes	15 min-utes	Average	Ratio B/A
Phenol:																				
A regular test....	80	90	100	90.0	1.22	70	75	80	75.0	1.02	55	60	60	58.3	1.20	45	50	50	48.3	1.14
B "washed" bacteria.........	100	110	120	110		70	80	80	76.6		60	70	80	70.0		50	55	60	55 0	
2,4,6-trichloro-phenol in the presence of alcohol:																				
A regular test....	2,000	2,250	2,500	2,250	2.37	1,000	1,250	1,250	1,166	1.16	1,250	1,500	1,500	1,417	2.23	600	700	700	666	1.10
B "washed" bacteria.........	5,000	5,000	6,000	5,333		1,250	1,250	1,500	1,333		3,000	3,000	3,500	3,166		600	800	800	733	
2,4,6-trichloro-phenol:																				
A regular test....		2,000	2,000	2,000							2,500	2,750	2,750							
B "washed" bacteria.........	4,000	4,500	5,000	5,000																
4,6-dichloro-resorcinol:																				
A regular test....	250	300	300	283	2.47	100	175	200	158	1.00	175	250	250	225	1.48	80	100	125	102	1.23
B "washed" bacteria.........	550	750	800	700		100	175	200	158		250	350	400	333		80	140	160	126	

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Table 5 shows the maximum dilutions of the substances tested which kill *B. typhosus* and *Staph. pyogenes-aureus* previously washed with a physiological salt solution. These dilutions are compared with the practical dilutions of the regular tests. The ratio columns indicate the relationship between the dilutions required to kill "washed" and "not washed" bacteria.

It appears first that the presence of the minute quantity or organic matter in the media influences the germicidal action of phenol only slightly. If the germicidal efficacy of phenol against *B. typhosus* and *Staph. pyogenes-aureus* is regarded as 1 in the regular test, then the phenol seems to be 1.22 times more effective against *B. typhosus* and 1.20 times more effective against *Staph. pyogenes-aureus* in the absence of media. Considerably greater differences are observed in the cases of the other two substances. Thus tri-chloro-phenol seems to show more than twice the efficacy against both *B. typhosus* and *Staph. pyogenes-aureus* when the minute quantity of the organic matter from the media is removed. Similar conditions are encountered in the case of di-chloro-resorcinol.

Referring to tri-chloro-phenol, the same remarks hold as given before, viz., the tests in the second horizontal column were carried out in the presence of alcohol, which fact may detract from the objective value of our findings. However, it appears from the tests in the third horizontal column, which refer to tests carried out with a saturated aqueous solution of tri-chloro-phenol, that the difference between tests carried out in the presence and those in the absence of alcohol, cannot be regarded as important. The blank spaces in the table indicate that in these particular cases, the saturated aqueous solution of tri-chloro-phenol (approximately 1:2000) was not sufficiently strong to effect a killing of the germs.

It is also of interest to consider the experiments which were carried out using "washed" bacteria with the subsequent addition of standard organic matter, as required by the specifications of the Hygienic Laboratory method. It appears that in practically all cases the dilution of disinfectant required to kill the "washed" germs in the presence of standard organic matter corresponds

very closely to the concentration required to kill "untreated" germs such as are used in the regular working procedure. From this two conclusions can be drawn:

1. Centrifuging did not impair the viability of the germ because under the conditions of the regular test, the "washed" germ showed the same resistance against the disinfectants tested as the untreated germ.[5]

2. The fact that the germicidal efficacy against "washed" germs is greater is owing practically entirely to the removal of the minute quantity of organic matter, normally present in 0.5 cc. of the culture which is brought in contact with the diluted disinfectant.

Finally another experiment was carried out in order to prove that the small quantity of organic matter present in the media is responsible for the considerable reduction of germicidal efficacy of certain compounds. A comparison of the following two cases suggested itself: In one case the quantity of media which is normally present in the amount of culture used is first brought into contact with the dilute disinfectant and subsequently with a culture previously freed from its media. In the other case the microörganism, freed from its media, is brought into contact with the dilute disinfectant first, and the media added subsequently. If the small quantity of organic matter present in the media actually interferes with the germicidal action of the disinfectant, then in the first experiment the effect would have to be less than in the second; it is understood, of course, that the microörganisms are exposed to the action of the disinfectant for the same period of time in both cases.

The process used is as follows: The culture is freed from its media by centrifuging and treating with a physiological salt solution as described before. In one case ("A") 0.5 cc. of media is added to 5 cc. of diluted disinfectant and allowed to stand three minutes, whereupon 0.5 cc. of "washed" culture is introduced. From this time on the procedure corresponds to that of our regu-

[5] According to Winslow and Brooke (1927), the viability of certain germs is impaired when suspended in distilled water while others are much less sensitive to this treatment.

lar standard bacteriological technique, i.e., one loopful is removed from this mixture and transplanted into fresh media after five, ten and fifteen minutes of contact respectively. In the other case ("B") 0.5 cc. of a suspension of culture in physiological salt solution is added to 5 cc. of disinfectant. After three minutes 0.5 cc. of media is introduced and two minutes later the first transplant is made in fresh broth; the following transplants are made five and ten minutes after the first transplant. Thus, also in this case, the bacteria were in contact with the disinfectant for five, ten and fifteen minutes respectively. In other words, the purpose of this experiment was to obtain a temporal separation of the two factors, namely germ and media, and to subject

TABLE 6

	B. TYPHOSUS					STAPH. AUREUS				
	Time of exposure				Increase of efficacy	Time of exposure				Increase of efficacy
	5 minutes	10 minutes	15 minutes	Average		5 minutes	10 minutes	15 minutes	Average	
					per cent					*per cent*
Phenol:										
A............	80	90	100	90	22	50	60	70	60	17
B............	100	110	120	110		65	70	75	70	
4,6-dichloro-resor-cinol										
A............	325	375	400	366	64	175	200	250	208	20
B............	550	600	650	600		225	250	275	250	

them to the influence of the disinfectant solution at different times and in different sequences instead of simultaneously.

The experiments were carried out with both *B. typhosus* and *Staph. pyogenes-aureus* using both phenol and dichlororesorcinol as germicides. Table 6 illustrates the conditions encountered.

It appears that there is a very considerable increase in the efficiency of dichlororesorcinol against *B. typhosus* when the operations are carried out according to scheme described under "B." The difference observed in the case of *Staph. progenes-aureus* is much less pronounced. In the case of phenol, no great difference between the two procedures could be expected on the basis of our previous experiments and none was found.

NOTES ON THE PREPARATION OF THE CHEMICAL COMPOUNDS USED IN THIS WORK

The substances used in this work were either prepared in our laboratory or purchased from the Eastman Company. All substances bought were carefully examined for purity, either by a determination of the melting point or by a halogen determination or both.

The determination of halogen was carried out in all cases according to Pregl's micro-modification of Carius' method. In those cases where the purity was not satisfactory for our purposes a further purification was carried out by redistillation or recrystallization.

Following are notes on the preparation of those chemicals which were made in our laboratory.

Preparation of 4-chloro-thymol $C_6H_2(OH) \cdot CH_3 \cdot C_3H_7 \cdot Cl$

Thymol was dissolved in two parts of carbon tetrachloride. The theoretical quantity of sulfuryl chloride was added drop by drop, with stirring and cooling. The carbon tetrachloride solution was shaken with a 10 per cent sodium hydroxide solution which dissolved the chlorothymol. The alkaline solution was separated from the carbon tetrachloride, washed with ether in order to remove traces of carbon tetrachloride and acidified. The precipitated oily chlorination product was washed with water, dried with sodium sulphate and distilled in a vacuum (5 mm.). The fraction boiling at 110 to 120° solidified. It was remelted and stirred with petroleum benzene until cold. Melting point (uncorrected) 60°, (lit. 59–60°).

Analysis: Substance, 13.284 mgm.: AgCl, 9.840 mgm. Calculated for $C_{10}H_{13}OCl$: Cl, 19.21. Found: Cl, 18.32.

Preparation of 4-bromo-thymol $C_6H_2(OH) \cdot CH_3 \cdot C_3H_7 \cdot Br$

Thymol was dissolved in two parts of glacial acetic acid. The theoretical quantity of bromine, dissolved in an equal part of glacial acetic acid, was added drop by drop with stirring and cooling. The acetic acid solution was poured into a large quantity of water. The precipitated bromination product was washed

with a 5 per cent solution of sodium carbonate and with water, dried over sodium sulphate and distilled in a vacuum. The fraction boiling between 120 to 135° solidified. It was remelted and stirred with petroleum benzene until cold. Melting point (uncorrected) 56°, (lit. 54–56°).

Analysis: Substance, 9.615 mgm.: AgBr, 7.962 mgm. Calculated for $C_{10}H_{13}OBr$: Br, 34.88. Found: Br, 35.25.

Preparation of 4-bromo-carvacrol $C_6H_2(OH) \cdot CH_3 \cdot C_3H_7 \cdot Br$

Carvacrol was dissolved in an equal volume of glacial acetic acid. The theoretical quantity of bromine, dissolved in one part of glacial acetic acid, was added drop by drop with stirring and cooling. The acetic acid solution was poured into a large quantity of water. The precipitated crude product was washed with a 5 per cent sodium carbonate solution and with water, dried over sodium sulphate and distilled in a vacuum. The vacuum distillation was repeated and the fraction boiling between 112 to 114° (5 mm.) was isolated.

Analysis: Substance, 11.989 mgm.: AgBr, 9.856 mgm. Calculated for $C_{10}H_{13}OBr$: Br, 34.88. Found: Br, 34.97.

Preparation of 4-chloro-resorcinol $C_6H_3(OH)_2Cl$

This compound was prepared according to Reinhard's (1878) method except for the purification, which was done by recrystallization from carbon tetrachloride instead of by sublimation. Melting point (uncorrected) 89.5°, (Reinhard 89°).

Analysis: Substance, 8.973 mgm.: AgCl, 8.812 mgm. Calculated for $C_6H_5O_2Cl$: Cl, 24.53. Found: Cl, 24.30.

Preparation of 4,6-dichloro-resorcinol $C_6H_2(OH)_2Cl_2$

This compound was prepared by treating a solution of resorcinol in ether with a 6 per cent solution of chlorine in carbon tetrachloride, using an excess of 5 per cent of chlorine over the amount of resorcinol. After the formation of hydrogen chloride fumes had subsided an air current was passed through the mixture in order to remove most of the solvent and of the hydrogen chloride. The remaining concentrated solution was decolorized

by boiling with Darco and filtered.   The crude product which crystallized from the filtrate was purified either by vacuum sublimation or by recrystallization from benzene.   Melting point (uncorrected) 109°.   Yield 75 per cent.

The attempt to prepare this compound according to the method of Reinhard (1878) was unsuccessful.   No consistent results could be obtained.   The melting point observed by Reinhard was 77°.

Analysis: Substance, 9.965 mgm.: AgCl, 16.162 mgm.   Calculated for $C_6H_4O_2Cl_2$: Cl, 39.62.   Found: Cl. 40.12.

Preparation of 2,4,6-trichloro-resorcinol $C_6H(OH)_2Cl_3$

This preparation was made according to the method of Benedikt (1884).   The substance was purified by recrystallization from boiling water and subsequently from carbon tetrachloride. Melting point 73.5° (uncorrected) in contrast to 83° as reported by Benedikt.

Analysis: Substance, 7.599 mgm.: AgCl, 15.349 mgm.   Calculated for $C_6H_3O_2Cl_3$: Cl, 49.84.   Found: Cl, 50.00.

Preparation of 4-bromo-resorcinol $C_6H_3(OH)_2Br$

This compound was prepared from 5-bromo-resorcylic acid $C_6H_2(OH)_2 \cdot COOH \cdot Br$ according to the method by Zehenter (1888, a).   The bromo resorcylic acid was prepared by bromination of β-resorcylic acid according to Zehenter's method (1888, b). The bromo resorcinol was purified by recrystallization from carbon tetrachloride.   Melting point 102° (uncorrected) (Zehenter reports 91°).

Analysis: Substance, 6.620 mgm.: AgBr, 6.522 mgm.   Calculated for $C_6H_5O_2Br$: Br. 42.28.   Found: Br, 41.92.

Preparation of 4,6-dibromo-resorcinol $C_6H_2(OH)_2Br_2$

This substance was prepared according to the method by Zehenter (1888, c).   The product was recrystallized from carbon tetrachloride and from water.   Melting point 115.5° (uncorrected) (Zehenter reports 110–112°).

Analysis: Substance, 5.801 mgm.: AgBr, 8.176 mgm.   Calculated for $C_6H_4O_2Br_2$: Br, 59.66.   Found: Br, 59.98.

Preparation of 2,4,6-tribromo-resorcinol $C_6H(OH)_2Br_3$

In the preparation of this substance the method described by Benedikt (1884) was followed rather closely.

The tribromoresorcinol was purified by recrystallization from water and from carbon tetrachloride. Melting point (uncorrected) 112° (Benedikt reports 111°).

Analysis of substance, 6.689 mgm.: AgBr, 10.899 mgm. Calculated for $C_6H_3O_2Br_3$: Br, 69.13. Found: Br, 69.34.

REMARKS

It appears from our experiments that there exists not only a relationship between the chemical constitution and germicidal action of certain groups of phenol and resorcinol derivatives, but that a definite quantitative relation is also to be found between the chemical constitution of these compounds and the impairment of their germicidal efficacy by organic matter. It is noteworthy that a large quantity of organic matter is by no means necessary to demonstrate this impairment; even the exceedingly small quantity of organic matter present in the media in which the culture is grown suffices to produce considerable differences in the germicidal efficacy of certain compounds.

Although the experimental results of this paper do not as yet permit definite conclusions to be drawn regarding the processes which are responsible for this impairment of germicidal efficacy, the following will give an idea of the working hypothesis on the basis of which we expect to proceed in further work on this problem.

The concept of organic matter, as used in bacteriological phraseology, is rather indefinite from a chemical viewpoint. Both constituents of the so-called organic matter, namely, gelatin and peptone, are products of partial hydrolysis of protein and might, in the light of older and recent studies on the chemical constitution of proteins and their cleavage products, be regarded probably as mixtures of polypeptides, amino-acid anhydrides and amino acids. In any case, the composition of organic matter will differ qualitatively and quantitatively, depending upon the initial material used and the method and duration of protein

hydrolysis applied in the preparation of these constituents of organic matter.

It is assumed that the impairment of the germicidal action of the compounds studied in this paper will be influenced by the presence of certain free reactive groups in these more complex protein cleavage products, such as the NH_2, $COOH$, OH, NH and SH groups; however, in contrast to the ideas of investigators whose work has been mentioned in the introduction, we believe that such impairment will not be brought about by a definite chemical reaction between the disinfecting compound and the "reactive" group, but rather by the formation of "additive" compounds in which, as in Werner's compounds, the secondary valencies will play an important rôle.   Assuming for instance that the reactive group in the protein is the amino group, then the additive compound may be represented thus:

$$\text{Protein}\text{—}NH_2\ldots HO\cdot R$$

The degree of impairment of the disinfectant action of a phenol derivative by organic matter will depend upon the stability of the additive compound of the respective phenol derivative and the protein cleavage product bearing the reactive amino group. In this particular instance the stability will depend upon the affinity of the hydroxyl group or groups for the amino group or groups.   One factor, which will influence the affinity, will be the "acidity" of the hydroxyl group, which depends, e.g., upon the number of halogen atoms in the nucleus of the phenol derivative and the "basicity" of the amino group which depends, e.g., upon the amino acid or peptide which carries the amino group.

It is obvious that these ideas regarding the impairment of disinfectant action by reactive groups may be applied to the disinfecting action proper, since possibly groups in proteins which hinder disinfecting action, may bear resemblance to those which attach the disinfectant molecule to the cell.

Of course, this does not mean that the importance of other factors, such as distribution coefficient, solubility, surface tension, etc., is disregarded.   They probably relate to the physical phases of the process of disinfection which precede the chemical phase,

viz., the fixation of the hydroxyl group of the phenol or its derivative by a reactive group of the protoplasm by means of secondary valencies; such fixation may subsequently produce other changes, e.g., protein precipitation which is regarded by some as the true cause of disinfection by this class of compounds.

SUMMARY

1. The results of experiments upon the disinfectant action of phenol, resorcinol and their halogen derivatives, against *B. typhosus* and *Staph. pyogenes-aureus*, tend to indicate that there is a distinct relationship between the constitution of these derivatives and the impairment of their disinfectant action by organic matter.

2. It is shown that even the exceedingly small quantity of organic matter present in the original culture suffices to produce very considerable impairment of the germicidal efficacy of certain compounds.

3. A working hypothesis of disinfectant action is outlined, depending upon the assumption of formation of so called molecular (additive) compounds of the disinfectant agent with certain free reactive groups in the organic matter, or the protoplasm, respectively, which is to form the basis for further work on the problem.

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